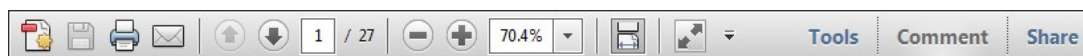
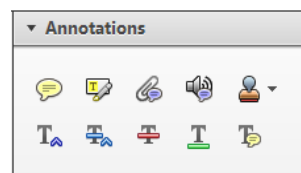


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1. [Replace \(Ins\)](#) Tool – for replacing text.

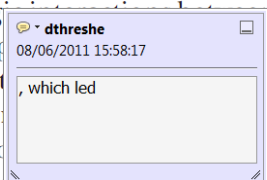


Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the [Replace \(Ins\)](#) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

standard framework for the analysis of microeconomic behavior. Nevertheless, it also led to the development of a new class of strategic form games. The number of competitors in the market is that the structure of the game is a main component. At the micro level, are exogenous variables and important works on entry by firms (M. Henceforth) we open the 'black box'.



2. [Strikethrough \(Del\)](#) Tool – for deleting text.



Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the [Strikethrough \(Del\)](#) icon in the Annotations section.

there is no room for extra profits as mark-ups are zero and the number of firms (set) values are not determined by Blanchard and ~~Kiyotaki~~ (1987), perfect competition in general equilibrium of aggregate demand and supply in the classical framework assuming monopoly between an exogenous number of firms.

3. [Add note to text](#) Tool – for highlighting a section to be changed to bold or italic.



Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the [Add note to text](#) icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

dynamic responses of mark-ups consistent with the **VAR** evidence

sation by Markov. The standard framework for the analysis of microeconomic behavior. The number of competitors in the market is that the structure of the game is a main component. At the micro level, are exogenous variables and important works on entry by firms (M. Henceforth) we open the 'black box'.



4. [Add sticky note](#) Tool – for making notes at specific points in the text.

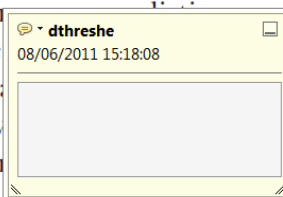


Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the [Add sticky note](#) icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

standard and supply shocks. Most of the standard framework for the analysis of microeconomic behavior. The number of competitors in the market is that the structure of the game is a main component. At the micro level, are exogenous variables and important works on entry by firms (M. Henceforth) we open the 'black box'.



5. **Attach File** Tool – for inserting large amounts of text or replacement figures.

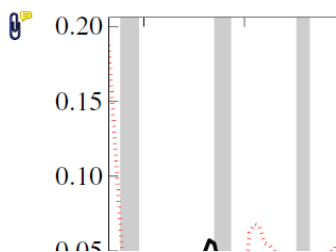


Inserts an icon linking to the attached file in the appropriate place in the text.

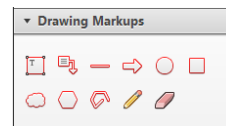
How to use it

- Click on the **Attach File** icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
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END

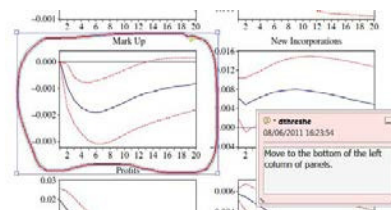


6. **Drawing Markups** Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks. Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.



How to use it

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- Double click on the shape and type any text in the red box that appears.



REVIEW ARTICLE

Ribosomally encoded antibacterial proteins and peptides from *Pseudomonas*

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Abstract

Members of the *Pseudomonas* genus produce diverse secondary metabolites affecting other bacteria, fungi or predating nematodes and protozoa but are also equipped with the capacity to secrete different types of ribosomally encoded toxic peptides and proteins, ranging from small microcins to large tailocins. Studies with the human pathogen *Pseudomonas aeruginosa* have revealed that effector proteins of type VI secretion systems are part of the antibacterial armamentarium deployed by pseudomonads. A novel class of antibacterial proteins with structural similarity to plant lectins was discovered by studying antagonism among plant-associated *Pseudomonas* strains. A genomic perspective on pseudomonad bacteriocinogeny shows that the modular architecture of S-pyocins of *P. aeruginosa* is retained in a large diversified group of bacteriocins, most of which target DNA or RNA. Similar modularity is present in as yet poorly characterized Rh (recombination hot spot) proteins and CDI (contact-dependent inhibition) proteins. Well-delimited domains for receptor recognition or cytotoxicity enable the design of chimeric toxins with novel functionalities, which has been applied successfully for S- and R-pyocins. Little is known regarding how these antibacterials are released and ultimately reach their targets. Other remaining issues concern the identification of environmental triggers activating these systems and assessment of their ecological impact in niches populated by pseudomonads.

Scope of the review

Pseudomonas strains are able to indwell very diverse niches, ranging from terrestrial and aquatic environments to tissues of eukaryotic hosts. Population of such environments involves a struggle for living space and organic nutrients with a plethora of other microorganisms. *Pseudomonads* display a highly versatile metabolism and several secondary metabolites affecting other bacteria and fungi have been identified, such as 2,4-diacetylphloroglucinol, phenazines, pyrrolnitrin, pyoluteorin, and lipopeptides (Gross & Loper, 2009). While such antibiotics mainly affect phylogenetically distant rivals, only few secondary metabolites are toxic to fellow pseudomonads that are attracted to common niches (Li *et al.*, 2011). The ribosomal machinery, on the other hand, enables biosynthesis of very diverse bacterial peptides and proteins, collectively designated bacteriocins, that are only deleterious to members of a certain bacterial species or a subset of phylogenetically close relatives of the

producer that itself carries an immunity protein-based system preventing self-intoxication. The study of such protein antibiotics has regained attention as a possible way to minimize collateral damage to nontarget microbiota and as a potential source of novel molecular targets to alleviate problems with multiresistance to available antibiotics.

Among Gram-negative bacteria, the colicins produced by enterobacteria are by far the best studied group of such narrow-spectrum antagonistic proteins, and a wealth of information is available on the molecular mechanisms involved in the different stages of their killing action (reviewed by Cascales *et al.*, 2007). Several bacteriocins of pseudomonads share basic characteristics with colicins and insights from colicin biology have been instrumental to identify novel so-called pyocins with similar cytotoxicities. Studies on interactions among Gram-negative bacterial relatives have disclosed in recent years that certain features of colicins and pyocins, such as the modular

nature of toxin/immunity pairs, are equally retained in substrates of Type V and Type VI systems that mediate antagonism (Braun & Patzer, 2013). In 2002, the review by Michel-Briand and Baysse was essentially confined to colicin-like and phage-like pyocins of *Pseudomonas aeruginosa*, as the only pseudomonad bacteriocins sufficiently characterized at that time. Here, we review the current knowledge of these systems and describe novel types of bacteriocins that have been identified in the last decade. This served as a basis to provide an overview of the bacteriocinogenic potential of the *Pseudomonas* genus by scrutiny of available (draft) genome sequences.

S-type pyocins

Soluble or S-type pyocins are protease- and heat-sensitive, chromosome-encoded bacteriocins from *P. aeruginosa* that are able to kill cells from the same species. These antibacterials are secreted as binary protein complexes consisting of a large protein that harbors the killing function and a smaller immunity protein that remains tightly bound to the cytotoxic domain of the former. The physical association of the toxin with its cognate immunity protein, reflected in the clustering of their structural genes, ensures that the producer strain is not harmed before the bacteriocin is released (Michel-Briand & Baysse, 2002).

To date, several S-type pyocins have been described and characterized: pyocins S1 (Ito *et al.*, 1970), S2 (Ohkawa *et al.*, 1973), AP41 (Holloway *et al.*, 1973; Sano & Kageyama, 1981), S3 (Duport *et al.*, 1995), S4 (Elfarash *et al.*, 2012) and S5 (Ling *et al.*, 2010). Pyocin Sa (Govan, 1986) turned out to be identical to pyocin S2 (Denayer *et al.*, 2007). A bacteriocin nearly identical to pyocin S1 but equipped with a different type of killing and immunity function was designated pyocin S6 (Dingemans *et al.*, 2013). Whereas all these proteins share identical parts or display significant local sequence homology, this is not the case for pyocin PaeM, which is produced without a known immunity partner (Barreteau *et al.*, 2009).

Although the study of S-type pyocins has mainly focused on their occurrence and action in the human opportunistic pathogen *P. aeruginosa*, genes encoding structurally related proteins are found in other *Pseudomonas* species as well, but functional characterization of the latter is lacking (Parret & De Mot, 2002).

Domain architecture of S-type pyocins

To kill a target cell, a S-type pyocin would first bind to a specific receptor located on the outer membrane of the bacterial cells and it would then be further translocated to exert its inhibitory function. The modular organization

design of the distinct domains correlates well with these multiple steps of mode of action (Michel-Briand & Baysse, 2002). In most S-type bacteriocins, the amino-terminal domain of the large protein bears the receptor-binding function and its carboxy-terminal part engenders the lethal effect (Sano *et al.*, 1993b; Parret & De Mot, 2002). In pyocins with a cytoplasmic target, they are connected by a domain that mediates translocation (corresponding to Pfam domain PF06958; Pyocin_S). An additional polypeptide segment of unknown function can be positioned between the receptor-binding and translocation domains, but this part is dispensable for killing, as observed for pyocins S2 and AP41 (Sano *et al.*, 1993a). The modular composition of S-type pyocins is also underlined by the fact that functional bacteriocins can be constructed by joining domains from different pyocins. Examples include combined domains of pyocins S1 and AP41 (Sano *et al.*, 1993a) and a pyocin S5/S2 chimera (Elfarash *et al.*, 2014). In addition, active pyocin/colicin hybrids with domains from pyocin S3 and colicin E3 (Gupta *et al.*, 2013; see Potential applications for S-type pyocins) and with domains from pyocins S1 or S2 and colicins E2 or E3 have been engineered (Kageyama *et al.*, 1996). Pyocin S5 and PaeM that do not require translocation to the cytoplasm for killing, exhibit a different domain architecture with the translocation domain preceding the receptor-binding domains (Barreteau *et al.*, 2009; Elfarash *et al.*, 2014). Pyocin size varies significantly, ranging from 289 amino acids for PaeM to 777 amino acids for AP41 (Table 1). A schematic representation of the gene and domain organization of characterized and selected predicted S-type pyocins in *Pseudomonas* spp. is shown in Fig. 1.

Biological properties and structure of S-type pyocins

Different S-pyocin-killing domains have been described in Table 1. Pyocin S1, S2, S3, and AP41 display DNase activity (Seo & Galloway, 1990; Sano, 1993; Sano *et al.*, 1993b; Duport *et al.*, 1995), while pyocin S4 harbors a tRNase (Elfarash *et al.*, 2012) and pyocin S6 a rRNase (Fig. 1; Dingemans *et al.*, 2013). Pyocin DNase domains typically bear a conserved HNH-endonuclease motif, as observed in pyocins S1, S2, and AP41 (Parret & De Mot, 2002). This motif constitutes the core of the catalytic site of the endonuclease and can chelate a single metal ion, required for hydrolysis of the dsDNA strand. These HNH-nucleases have also been detected in colicins (Papadakis *et al.*, 2012). Pyocin S3 on the contrary does not contain this HNH-motif and its lethal domain lacks sequence homology with the corresponding domains of other DNase pyocins (Parret & De Mot, 2002). Enzymatic

Table 1. Characteristics of *Pseudomonas aeruginosa* pyocins

Pyocin	Strain	Mode of action	Toxin		Immunity protein		
			Amino acids	Domain(s)*	Amino acids	Domain†	Receptor
AP41	PAF41-2	DNase (HNNH)	777	PF06958; <u>PF12639</u>	90	PF01320	?
M1	JJ692	Lipid II degradation	289	<u>PF14859</u>	–	–	?
S1	NIH-H	DNase (HNNH)	618	PF06958; <u>PF12639</u>	87	PF01320	?‡
S2	PAO1	DNase (HNNH)	689	PF03515; PF06958; <u>PF12639</u>	87	PF01320	FpvAI
S3	P12	DNase	766	PF06958	153	–	FpvAI
S4	PAO1	tRNase	764	PF03515; PF06958; <u>PF12106</u>	112	PF11480	FpvAI
S5	PAO1	Pore-forming	498	<u>PF01024</u>	108	PF03526	FptA
S6	CF-PA39	rRNase	571	PF06958; <u>PF09000</u>	77	–	?
M4	BL01	Lipid II degradation	342	<u>PF14859</u>	–	–	?
S7	BWHP5A018	rRNase	642	PF06958; <u>PF09000</u>	77	–	?
S8	VRFA07	DNase (HNNH)	772	PF06958; <u>PF12639</u>	85	PF01320	?
S9	BL04	DNase (HNNH)	421	PF06958; <u>PF12639</u>	84	PF01320	?
S10	PABL056	DNase	517	PF05488; PF06958	157	–	?
S11	LESB58	tRNase	662	PF03515; PF06958; <u>PF11429</u>	90	PF09204	?
S12	PA7	tRNase	740	PF06958; <u>PF11429</u>	90	PF09204	?

Proteins with demonstrated bacteriocin activity and representatives of predicted novel pyocin types are shown in bold and italic font, respectively.

*Pfam domains are ordered from amino- to carboxy-terminus: PF01024, Colicin; PF03515, Cloacin; PF05488, PAAR_motif; PF06958, Pyocin_S; PF09000, Cytotoxic; PF11429, Colicin_D; PF12016, Colicin_C; PF12639, Colicin-DNase; PF14859, Colicin_M. The carboxy-terminal domain conferring cytotoxic activity is underlined.

†Pfam domains: PF01320, Colicin_Pyocin; PF03526, ImmE1; PF09204, Colicin_immun; PF11480, ImmE5.

‡The receptor of pyocin S1 is unknown, but is FpvA independent (Denayer *et al.*, 2007).

activity has not been detected for pyocin S5; instead, this pyocin is known to kill a target bacterium via pore formation, resulting in membrane damage and leakage of intracellular compounds (Fig. 1; Ling *et al.*, 2010).

A pyocin with yet a different cytotoxic activity was identified in *P. aeruginosa* strains JJ692 and DET08 encoded within their *exoU*-carrying genomic islands. This enzyme, PaeM, displays homology with colicin M, a lipid II-degrading bacteriocin from *Escherichia coli*. It does not exert its enzymatic activity in the cytoplasm; instead, it is active in the periplasm where it blocks peptidoglycan synthesis (Fig. 1; Barreateau *et al.*, 2009). Unlike the lytic activity of colicin M on sensitive *E. coli* cells, PaeM only exhibits a bacteriostatic effect on its target cells (Barreateau *et al.*, 2009). For the plant pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 and the wheat rhizosphere isolate *Pseudomonas fluorescens* Q8r1–96, recombinant homologs (PsyM and PflM, respectively) were shown to be functional toxins as well, however, displaying lower *in vitro* phosphodiesterase activity. Of fourteen *P. aeruginosa* strains tested, only two strains were susceptible to PaeM and one of these indicators also was inhibited by PflM. No other target strain was identified among 40 strains representing the three species, pointing to a quite narrow activity spectrum for this type of pseudomonad bacteriocin, as compared to S-type pyocins. In a subsequent independent study, activity of the DC3000 recombinant enzyme, there denoted syringacin M, was shown against two *P. syringae* strains belonging to the pathovars *syringae*

(LMG 5084) and *lachrymans* (LMG 5456), while other *Pseudomonas* species were not affected. The same narrow activity spectrum was exhibited by a close homolog purified from mitomycin-induced culture supernatant of *P. syringae* pv. *syringae* LMG 1247 (Grinter *et al.*, 2012b).

Currently, structures of only two pyocins have been solved: PaeM from *P. aeruginosa* and syringacin M from *P. syringae* (Fig. 2). These proteins share structural similarities with colicin M. Their crystal structures consist of a short disordered amino-terminal translocation domain, followed by a central globular α -helical receptor-binding domain and a cytotoxic domain incorporating a half β -barrel fold, characteristic for these bacteriocins (Zeth *et al.*, 2008) but adopting a modified active site architecture. A Mg^{2+} ion was identified in the active site of PaeM in line with the dependence of colicin M activity on this cation (Barreateau *et al.*, 2012). However, in crystallized syringacin M, this site was occupied by Ca^{2+} that supports catalytic activity similarly to Mg^{2+} (Grinter *et al.*, 2012b). Barreateau and coworkers (Barreateau *et al.*, 2012) showed that *in vitro* catalytic activity of the isolated cytotoxic domain was enhanced, suggesting that interdomain interactions dampen the enzymatic activity which is supposed to be fully released upon cellular entry and interaction with its substrate. Although the syringacin M receptor-binding domains lacks discernible sequence homology to the equivalent colicin M region, they adopt a very similar fold, presumably as a result of diversifying selection (Grinter *et al.*, 2012b) rather than recombination (Zeth

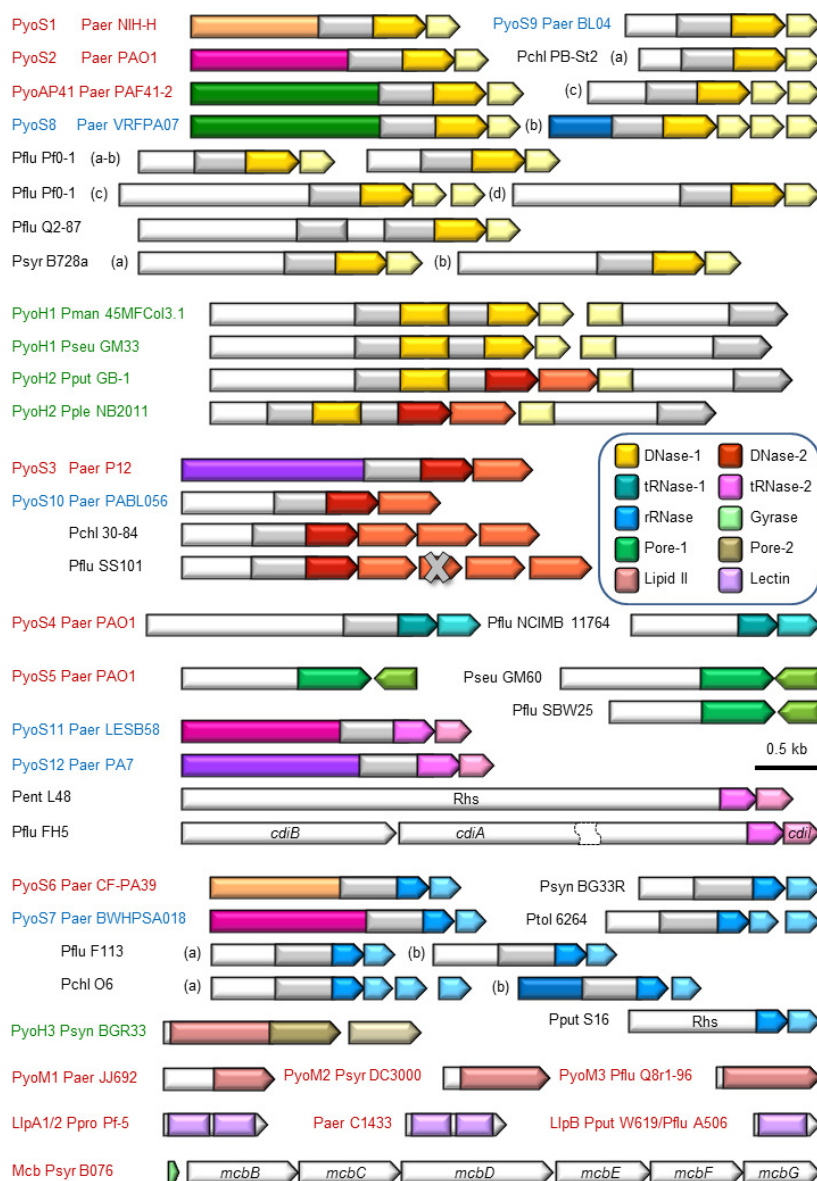
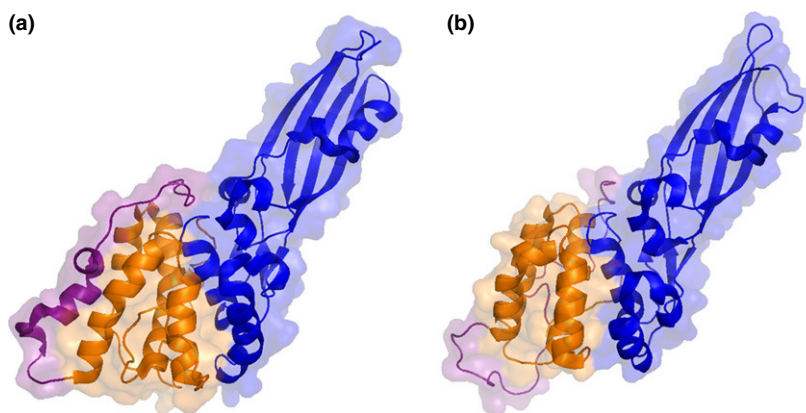


Fig. 1. Gene organization of pseudomonad bacteriocins. Arrows illustrate representative coding regions and are grouped according to known or predicted toxic activity. The DNA regions encoding the respective bacteriotoxic domains are highlighted in color as specified in the inset box. The coding region(s) of the respective immunity protein(s) located downstream of the toxin gene, if present, are shown in a fainter shade of the same color. The Pfam accession numbers of killing and immunity domains are specified in Table 1. The Pyocin_S domain is shown in gray. The additionally detected toxin/immunity domains in putative bacteriocins are marked as Pore-2 (PF01024/PF03857) and tRNase-2 (PF11429/PF09204). Additional colors indicate conservation of an amino-terminal domain between different bacteriotoxic proteins and noncolored parts denote a lack of significant similarity. Genomic DNA regions with clustered bacteriocin genes and multiple bacteriocin operons within a single strain are differentiated with labels (a) through (d). Names of functionally characterized bacteriocins are highlighted with red font. Proposed names for new types of pyocins with a single toxic domain (blue) or hybrid forms with two toxic domains (green) are also shown in color. The crossed region represents a pseudogene. The scale bar represents a DNA region of 500 bp. Abbreviations used for species names: Paer, *Pseudomonas aeruginosa*; Pchl, *Pseudomonas chlororaphis*; Pent, *Pseudomonas entomophila*; Pflu, *Pseudomonas fluorescens*; Pman, *Pseudomonas mandelii*; Pple, *Pseudomonas plecoglossicida*; Ppro, *Pseudomonas protegens*; Pput, *Pseudomonas putida*; Pseu, *Pseudomonas* sp.; Psyn, *Pseudomonas synxantha*; Psyr, *Pseudomonas syringae*; Ptol, *Pseudomonas tolaasii*. Other abbreviations: PyoS, pyocin S; Rhs, protein with Rhs domain; *cdiBAI*, CDI system operon; *mcbABCDEFG*, B-type microcin operon. An overview of representative tailocin gene clusters is presented in Fig. 6.

COLOR

Fig. 2. Structures of lipid II-degrading M-type pyocins PaeM (a, PDB 4G75) and syringacin M (b, PDB 4FZM). Ribbon structures are shown in a transparent surface model. Translocation domains are colored purple, receptor-binding domains orange and catalytic domains blue. The amino-terminal translocation domain of syringacin M is unstructured.



et al., 2008). Taking into account that these colicin M-like proteins form a distinct group of pyocins as compared to other modular S-type bacteriocins, we propose pyocin M to refer to this *Pseudomonas* protein family.

Extracellular release of S-pyocin/immunity protein complexes

S-type pyocins are released as bacteriocin/immunity protein complexes at equimolar ratio. The size of the immunity proteins of S-type pyocins ranges from 77 to 153 amino acids, much smaller than the cognate killer proteins (Table 1). Their coexpression is crucial as they transiently inhibit the lethal function of the pyocin (Sano & Kageyama, 1981; Seo & Galloway, 1990; Sano *et al.*, 1993b; Duport *et al.*, 1995; Rasouliha *et al.*, 2013). Immunity proteins from S1, S2 and AP41 share homology as they shield homologous HNH nuclease domains. Pyocin S3 is associated with a different type of immunity protein, in line with its classification as a different type of DNase toxin (Parret & De Mot, 2002). Protection is provided by interaction of the amino-terminal end of the immunity protein and the carboxy-terminal cytotoxic part of the killer protein (Sano *et al.*, 1993b; Kageyama *et al.*, 1996). Bacteriocin-producing cells may need to neutralize pyocin produced by other clonemates. It was found that this so-called soaking effect causes a reduced fitness of the pyocin producer strain (Inglis *et al.*, 2013).

To ensure a swift 'trapping' of a corresponding killing domain, immunity genes of nuclease-type of bacteriocins are located immediately downstream of the pyocin genes and transcribed as an operon (Fig. 1). Their Shine–Dalgarno boxes are located within the lethal part of the pyocin gene (Sano *et al.*, 1993b). Interestingly, the presence of 'orphan' immunity genes, encoding proteins that complement a matching S-type pyocin toxicity domain but that are not preceded by toxin genes, confers protection to sensitive strains. This way, invading pyocins

may be captured, impeding their action in target cells (Denayer *et al.*, 2007; Elfarash *et al.*, 2012; Rasouliha *et al.*, 2013; Elfarash *et al.*, 2014). Notably, the immunity gene of the pore-forming pyocin S5 is transcribed in the opposite direction of the toxin gene (Fig. 1; Stover *et al.*, 2000).

The mechanism behind self-immunity of pseudomonads producing colicin M-like bacteriocins is currently not known. In *E. coli*, this is ensured by a coexpressed immunity protein (Cmi) anchored in the cytoplasmic membrane facing the periplasm (Cascales *et al.*, 2007). Genes encoding homologs of Cmi or the structurally related YebF protein (Gérard *et al.*, 2011; Usón *et al.*, 2012) are not found in *Pseudomonas* genomes.

After synthesis, the pyocin complexes are released from the producers, without the need of a (cleavable) signal sequence. Colicins take advantage of a lysis protein, encoded nearby the colicin gene, whereas S-type pyocins do not have these available (Michel-Briand & Baysse, 2002). It was suggested that S-pyocins may take advantage of the lytic systems enabling the secretion of phage tail-like bacteriocins (see Genetic determinants of R-type pyocins; Nakayama *et al.*, 2000). However, several S-pyocin-encoding *Pseudomonas* strains lack phage tail-like bacteriocin clusters and their accompanied release cassettes (see *In silico* analysis of tailocins in other *Pseudomonas* species).

S-type pyocin receptors and translocation

The observation that several S-type pyocins kill target cells much more efficiently under iron-poor conditions led to the idea that these bacteriocins take advantage of iron-regulated receptors for cell entry (Ohkawa *et al.*, 1980; Sano *et al.*, 1993b; Duport *et al.*, 1995; Elfarash *et al.*, 2012, 2014). When iron is limiting, bacteria will express outer-membrane proteins that promote its uptake. One important strategy used by *P. aeruginosa* to

enhance iron uptake is the secretion of the siderophores pyoverdine and pyochelin, low-molecular-weight Fe^{3+} -chelating molecules. Three pyoverdine types can be distinguished, classified based on their oligopeptide side chains and each recognized by a specific outer-membrane receptor. After iron binding, siderophores are taken up by the iron-regulated outer-membrane proteins (IROMPs). Energy for this process and siderophore recycling is transduced by the cytoplasmic membrane protein TonB (Cornelis & Dingemans, 2013).

Mutagenesis experiments provided unequivocal evidence that several S-type pyocins indeed use IROMPs as a receptor (Table 1). Pyocin S2 and S4 take advantage of the type I ferripyoverdine receptor (FpvAI; Ohkawa *et al.*, 1980; Smith *et al.*, 1992; Denayer *et al.*, 2007; Elfarash *et al.*, 2012), whereas pyocin S3 uses the type II ferripyoverdine receptor (FpvAII; Baysse *et al.*, 1999). The pore-forming pyocin S5 hijacks the FptA ferripyochelin receptor to inhibit sensitive strains (Elfarash *et al.*, 2014). As the latter receptor is widespread among *P. aeruginosa* strains, the percentage of sensitive strains in a representative test panel is higher than usual. The receptors of pyocins S1 and S6 have not yet been identified, but sensitivity seems independent of the ferripyoverdine receptor produced (Denayer *et al.*, 2007; Dingemans *et al.*, 2013).

Following contact with these receptors, pyocins are translocated across the outer membrane. The exact mechanism remains unknown though the use of the ferrisiderophore receptors suggests that S-type pyocins are translocated in a similar way as the pyoverdines and pyochelin, energized by the TonB system (Cornelis & Dingemans, 2013). Several colicins are translocated via the TonB machinery as well, equally taking advantage of outer-membrane receptors for siderophores, such as linear catecholate transporter Cir, iron-enterobactin transporter FepA and ferrichrome-iron receptor FhuA (Jakes & Cramer, 2012).

Pyocin AP41 seems to enter cells via another receptor and uptake mechanism, although iron was found to play a major role as well. Different mutants tolerant to pyocin AP41 (tol phenotype) were isolated (Holloway *et al.*, 1973), revealing involvement of a cluster of seven genes, organized in three operons (*orf1-tolQRA*, *tolB* and *oprL-orf2*) and regulated by iron availability and growth phase (Duan *et al.*, 2000). The *orf1* gene is nonessential, while mutants in *tolQ* and *tolA* could not be obtained, probably because gene inactivation would result in a lethal phenotype (Wei *et al.*, 2009). Nevertheless, introduction of the *tolQRA* genes in a *tol* mutant is able to restore killing by AP41 (also called AR41), indicating that the Tol proteins are involved in pyocin uptake (Dennis *et al.*, 1996). High iron concentration (FeCl_3) causes a reduced expression of the complete locus, due to the presence of a Fur-repressor

(Lafontaine & Sokol, 1998; Duan *et al.*, 2000). In contrast, RegA functions as a positive regulator under iron-restricted conditions (Duan *et al.*, 2000). Study of the expression profile of *orf1-tolQRA* demonstrated that this operon contains one constitutive promoter in front of *orf1*, and one iron-regulated promoter located within *orf1* (Wei *et al.*, 2009). The *oprL* gene encodes a 18 kDa outer-membrane peptidoglycan-associated lipoprotein required for a normal cellular morphology and is located downstream of *tolB*. An *oprL* knockout mutant is viable but is very sensitive to osmotic pressure, underlining its role in cell envelope integrity (Lim *et al.*, 1997). More recently, the growth phase-dependent regulation of the *tol-oprL* locus was found to be controlled by quorum sensing (QS), dependent on *N*-acyl homoserine lactone (AHL). When residing in a stationary phase, bacteria will not opt for a pronounced expression of these genes as they are required for growth and cell division, explaining the downregulation of the locus (Wei *et al.*, 2009). Although the receptor of pyocin AP41 is not known, the proposed uptake route via the Tol machinery is in line with observations made for colicins. In Tol-energized uptake of several colicins, the primary outer-membrane receptors (such as the TonB-dependent vitamin B_{12} transporter BtuB) and the outer-membrane proteins actually translocating the bacteriocins (such as the porin OmpF) are not involved in siderophore-mediated iron uptake (Jakes & Cramer, 2012).

Potential applications for S-type pyocins

The S-type pyocins may be valuable tools in future therapeutic applications. This is illustrated by the potent activity of pyocin S2 against *P. aeruginosa* biofilms. Tested against different clinical isolates, this bacteriocin kills both mucoid and nonmucoid strains with similar efficiency when these are growing in a biofilm. Moreover, survival rates of biofilms treated with tobramycin or aztreonam at equal concentrations are considerably higher (> 100-fold). *In vivo* pyocin S2 activity was validated in a *P. aeruginosa*-infected *Galleria mellonella* caterpillar model (Smith *et al.*, 2012).

From synthetic biology, engineered *E. coli* strains were developed, able to sense the presence of *P. aeruginosa* cells and subsequently killing them, both in planktonic and biofilm growth conditions. In a first approach, *E. coli* was equipped with a pyocin S5 gene, under the control of a *luxR* promoter (Saeidi *et al.*, 2011). The latter is activated after binding of a LasR/3-oxo- C_{12} -AHL complex. In the construct, constitutive expression of *lasR* gene was driven by a *tetR* promoter. Hence, production of 3-oxo- C_{12} -AHL, a native AHL from *P. aeruginosa*, can trigger biosynthesis of pyocin S5, concomitant with the accumulation of

colicin E7 lysis protein, put under control of the same P_{las} promoter. When reaching a threshold intracellular concentration of E7 lysis protein, *E. coli* cells will burst, causing the release of the pyocin into the environment and killing *P. aeruginosa* (Saeidi *et al.*, 2011). In a similar system, *E. coli* sentinels were armed with a chimeric pyocin, constituted with the receptor and translocation domain of pyocin S3 and the killing and immunity domain of colicin E3, under the control of the P_{las} promoter (Gupta *et al.*, 2013). The target detection module provides LasR, specifically interacting with the *P. aeruginosa* autoinducer. A P_{las} -regulated *gfp* gene was included for fluorescence-based monitoring of the binding of 3-oxo-C₁₂-AHL to the regulatory protein. To enable secretion rather than suicidal release of the chimeric toxin, the flagellar secretion tag FlgM was introduced. On semi-solid medium, the *E. coli* producer, insensitive to its own engineered toxin, inhibited the growth of co-cultured *P. aeruginosa*. In principle, the modular design of these sensitive sense and destroy systems allows to virtually target any pathogen of interest, provided that an appropriate bacteriocin gene is known. Especially, when sentinels are equipped with multiple toxins, targeting different cellular receptors, resistance of a bacterial target can be overcome.

In silico analysis reveals novel S-type pyocins in *Pseudomonas* genomes

So far most of the work on S-type pyocins has been focused on *P. aeruginosa*, relying on the functional characterization of growth-inhibitory proteins detected in supernatants or in agar. *In silico* analysis previously suggested that these antibacterials may be far more widespread among pseudomonads (Parret & De Mot, 2002). In the past few years, decreasing costs of sequencing led to an exponential growth in available (draft) genome sequence data. In this section, we highlight the distribution and diversity of S-type pyocinogenic genes in *P. aeruginosa* and other pseudomonads. This analysis revealed the existence of several novel S-type pyocin genes in *Pseudomonas* genomes.

HNH DNase pyocins

DNA degradation by pyocin S1, S2, and AP41 is mediated by their carboxy-terminal nuclease domain (HNH family; Pfam domain PF12639, SMART domain SM00507). Pyocins S1 and S2 have virtually identical DNase domains (apart from a single conservative Val-Ile substitution), but they share only *c.* 60% amino acid sequence identity with the corresponding part of pyocin AP41. This is reflected in the homology of their immunity proteins: those of pyocin S1 and pyocin S2 differ by

only one amino acid but they share only *c.* 44% identity with the one of pyocin AP41. Current genome sequence data suggest a prevalence of genes encoding orthologs of pyocin S1 (23 strains) or pyocin AP41 (17 strains) over those encoding pyocin S2 proteins (11 strains; Supporting information, Table S1). AP41 and S1 pyocinogeny are combined in seven strains, significantly more than the pyocin AP41-S2 combination (two strains), whereas no strain carries both pyocin S1 and S2 genes. Probably, the occurrence of pyocin AP41 on a transposable element (TnAP41) has promoted its acquisition by various *P. aeruginosa* strains (Sano & Kageyama, 1993).

Pseudomonas aeruginosa genome analyses uncovered two additional HNH subtypes present in a few strains only. In three pyocin S2-positive strains (BL09, BL13 and BL20) and strain VRFP07, the pyocin AP41 amino-terminal domain is fused to a distinct DNase domain that shares only *c.* 63% amino acid sequence identity with pyocins S1, S2, and AP41 (*c.* 44% for the associated immunity protein). For this third HNH-type protein, the designation pyocin S8 is proposed (Fig. 1, Table 1). A fourth divergent HNH subtype is found in strains BWHPSA026 (presence also combined with pyocin S2), BL04, HB15, and VRFP01. Its DNase domain bears more similarity to the previous type (60% amino acid identity vs. only 48% for pyocins S1, S2, and AP41), but it contains a different, much smaller amino-terminal domain (*c.* 200/350 residues less than in pyocins S1/AP41). This fourth type HNH pyocin is designated pyocin S9 here (Fig. 1, Table 1). The difference from the other *P. aeruginosa* pyocin systems is again reflected in a divergent cognate immunity protein.

Overall, pyocin S9 of *P. aeruginosa* is actually more similar (*c.* 50% amino acid identity) to a number of putative bacteriocins encoded in the genomes of strains related to the *P. fluorescens* clade such as *Pseudomonas chlororaphis* subsp. *aureofaciens* 30–84 (Loper *et al.*, 2012). These are part of a large group of *c.* 70 different putative bacteriocins that were retrieved through a search of pseudomonad genomic sequences for gene product pairs consisting of a protein with a HNH DNase-like carboxy-terminal domain and its apparent self-immunity-conferring partner. Phylogenetic analysis of a representative subset of these predicted bacteriocins visualizes the broad diversity collectively represented by these DNase domains composed of about 135 amino acids (Fig. 3). The distribution of most subclusters is not confined to particular species, as already noted for the functionally characterized and predicted bacteriocins occurring in *P. aeruginosa*. Even more sequence divergence is present among the corresponding immunity proteins that comprise on average about 85–90 amino acids (Fig. S1). Also, the amino-terminal domains of the killer proteins are

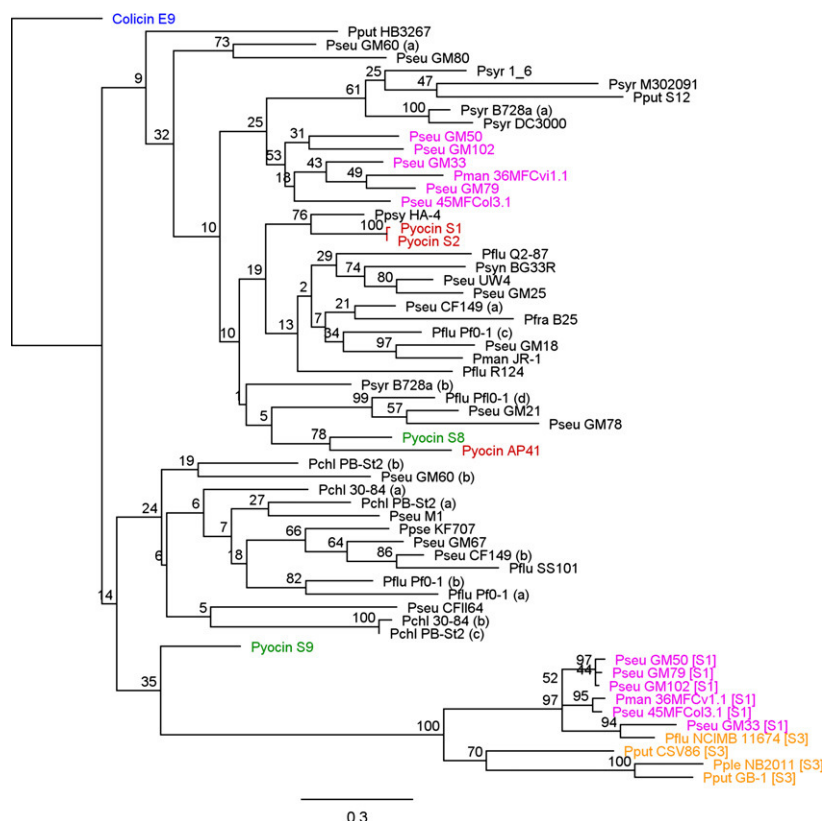


Fig. 3. Phylogenetic analysis of cytotoxic domains in representative pseudomonad bacteriocins of the HNH DNase pyocin family. The ML phylogenetic tree of HNH-related DNase domains is rooted with the corresponding *Escherichia coli* colicin E9 domain (blue). Multiple predicted bacteriocins occurring in a particular strain are marked by extensions (a) through (d). For *Pseudomonas aeruginosa*, the functionally characterized members are shown in red and the newly proposed pyocins types S8 and S9 are marked in green. Internal sequences present in dual-domain proteins with a carboxy-terminal domain of the same family [extension (S1)] or in combination with a carboxy-terminal domain related to pyocin S3 [extension (S3)] are highlighted in pink and orange, respectively. For the former type, the corresponding carboxy-terminal domains are shown in the same color. Abbreviations used for species names can be retrieved in the legend of Fig. 1. Additional abbreviations: Pfra, *Pseudomonas fragi*; Ppse, *Pseudomonas pseudoalcaligenes*; Ppsy, *Pseudomonas psychrophila*. The scale bar represents 0.3 substitutions per site. Bootstrap values (percentage of 100 replicates) are shown at the branches.

very diversified in sequence and length, ranging from c. 270 amino acids (for the majority of them) to 780 residues for *P. fluorescens* Q2–87, a wheat rhizosphere isolate with take-all biocontrol activity (Fig. 1; Loper *et al.*, 2012). Remarkably, the latter proteins harbor two Pyocin_S domains.

In some strains (e.g. *Pseudomonas* sp. GM25, *Pseudomonas pseudoalcaligenes* KF707), the first, probably, cognate immunity gene is followed by a second immunity gene that might protect against related DNase bacteriocins produced by rival pseudomonads. The capacity of a particular strain to encode multiple functionally related HNH-type pyocins seems not to be uncommon among pseudomonad species (Table S1). Most prominent in this respect is *P. fluorescens* Pf0–1 with four such systems, of which two are organized as a tandem of toxin/immunity gene pairs. The cluster of one of the other systems is expanded with

an additional immunity gene. The *P. chlororaphis* subsp. *aurantiaca* PB-St2 genome is equipped with the potential to encode three DNase bacteriocins of this type. In addition to the three respective immunity genes, this strain contains an extra immunity gene at a fourth genomic location that lacks an appropriate DNase partner (Fig. 1).

A dual DNase-based bacteriocinogenic capacity is also found in *P. syringae* pv. *syringae* B728a (Figs 1 and 3). The predicted toxins are of similar sizes (c. 650 amino acids) but lack pronounced sequence similarity, which also applies to the respective immunity proteins. Relatives of one of these systems occur in other *P. syringae* strains (e.g. *P. syringae* pv. *actinidiae* M302091, *P. syringae* pv. *tomato* DC3000), while the second one has a much more restricted distribution in this phytopathogenic species. In general, strains originating from soil and plant

environments, including some strains with biocontrol capacities such as *P. fluorescens* Q2-87 and SS101, *Pseudomonas synxantha* BG33R, and *P. chlororaphis* subsp. *aureofaciens* 30–84 (Loper *et al.*, 2012), are well represented in the pseudomonad collection displaying this kind of bacteriocinogeny. This is also apparent from the frequent occurrence of this antagonistic property in several isolates that were sequenced as part of a microbiome study of the poplar rhizosphere and endosphere (Brown *et al.*, 2012). Of 21 isolates, 11 strains (*Pseudomonas* sp. GM18, GM21, GM25, GM33, GM50, GM60, GM67, GM78, GM79, GM80 and GM102) are potentially able to produce one or two DNA-targeting bacteriocins of the HNH family.

Non-HNH DNase pyocins

The DNase domain of *P. aeruginosa* pyocin S3 lacks homology to the cytotoxic part of members of the HNH DNase pyocin family. Genome querying revealed gene pairs encoding putative bacteriocins of this type in at least 23 *P. aeruginosa* strains in addition to strain P12 (Table S1). Close orthologs are encoded by some strains such as the highly virulent *P. aeruginosa* UCBPP-PA14 (Lee *et al.*, 2006), whereas also more diverged homologs occur, for instance in the melon rhizosphere isolate *P. aeruginosa* M18 (Wu *et al.*, 2011a). A putative novel representative of this bacteriocin group, only showing significant homology within the carboxy-terminal nuclease domain, is apparently encoded by the blood stream isolate *P. aeruginosa* PABL056 (Ozer *et al.*, 2012) and is designated pyocin S10 (Fig. 1, Table 1). Notably, these strains lack the capacity to also produce a HNH-type of bacteriocin, with few exceptions: genes encoding pyocin S3 and the newly identified pyocin S9 are both present in strains BL04 and HB15, whereas the combination of pyocins S3 and AP41 genes only is found in strain M9A1.

Phylogenetic analysis of the pyocin S3-like cytotoxic domains derived from predicted bacteriocin genes present in a rather limited number of strains from other pseudomonad species, visualizes the extensive sequence diversity evolved for this module (Fig. S2). Compared with the S3 prototype from *P. aeruginosa* P12, the level of amino acid identity ranges from 73% (*P. aeruginosa* M18) down to 38% (*Pseudomonas putida* GB-1). The amino-terminal domains of these putative bacteriocins also vary markedly in size (between c. 300 and 750 amino acids). The degree of sequence conservation between the cognate immunity proteins is much lower compared with the respective DNase domains (Fig. S3). For instance, the modules of *P. aeruginosa* strains P12 and M18 share only 40% amino acid sequence identity, and alignment of the strain P12 and *P. putida* GB-1 sequences reveals only borderline

similarity (24% identity). Contrary to the observed preference of *P. aeruginosa* strains for deploying only one of both DNase-type bacteriocins, combined HNH/S3-type bacteriocinogenic potential is more frequent in other pseudomonad species. Among nine different S3-like systems identified outside the *P. aeruginosa* species, three were harbored by strains equally carrying genes for a member of the HNH family: *P. chlororaphis* subsp. *aureofaciens* 30–84 and *P. fluorescens* SS101, two wheat isolates with biological control potential for fungal diseases (Loper *et al.*, 2012), and the river isolate *Pseudomonas* sp. M1, of interest for its capacity to degrade recalcitrant organic compounds (Soares-Castro & Santos, 2013). Moreover, a novel type of hybrid bacteriocin protomer in which the regular carboxy-terminal pyocin S3-like module is combined with a centrally located HNH domain, is found in four additional strains (discussed in section Putative novel pyocins with a tandem DNase architecture).

In most of these strains, the characteristic genetic organization for nuclease bacteriocins is conserved, with a toxin gene immediately followed at its 3'-end by the cognate immunity gene. As already pointed out for the HNH family, in some S3 pyocinogens, the putative self-immunity gene is part of an expanded immunity locus, composed of additional immunity genes of the same type in a tandem organization (Fig. 1). Notable examples of expanded pyocin S3-related clusters are found in *P. chlororaphis* subsp. *aureofaciens* 30–84 and *P. fluorescens* SS101. In strain 30–84 a cluster of three such immunity genes is present. Their sequence similarity hints to a common origin and/or duplication events (Fig. S3). Even four immunity homologs are clustered in *P. fluorescens* SS101, but the second gene appears to be inactive due to a frameshift in the coding region (Fig. 1). The sequence similarity among these SS101 immunity gene products is quite low. The similarity of the fourth gene to a self-immunity gene present in another plant-associated pseudomonad (*P. syringae* pv. *avellanae* ISPaVe037; O'Brien *et al.*, 2012) suggests its acquisition by horizontal transfer rather than gene duplication (Fig. S3).

Some pseudomonads that lack an identifiable pyocin S3-type nuclease gene, do carry a S3-type immunity gene (Fig. S3). Such chromosomal orphan is present in the biocontrol strain *Pseudomonas protegens* Pf-5 isolated from soil (Loper *et al.*, 2012), while distinct but mutually related plasmid-borne genes are found in another biocontrol strain originating from pear phyllosphere (on plasmid pA506 of *P. fluorescens* A506; Stockwell *et al.*, 2013) and in a cave isolate (plasmid pMP-R124 of *P. fluorescens* R124; Barton *et al.*, 2013). Highly conserved orthologs of a related orphan gene designated *bip*, encoding a putative bacteriocin-immunity protein and residing on plasmid pPsv48B of the phytopathogen *Pseudomonas savastanoi*

NCPPB 3335 (Bardaji *et al.*, 2011), are located on plasmid pPSR1 of *P. syringae* pv. *syringae* A2 (Sundin *et al.*, 2004) and also occur in the genome of several other pathogens [pv. *glycinea* B076 (Qi *et al.*, 2011); pv. *maculicola* ES4326 (Schreiber *et al.*, 2012); pv. *theae* ICMP 3923]. In these plasmids, all members of the pT23A family, the bacteriocin/immunity gene is part of a small cargo region located between two backbone regions (replication/stable maintenance and MOB_{p6}-type conjugative transfer). This strategic positioning suggests that such bacteriocin/immunity genes can spread by conjugative plasmid transfer and could fulfill an ecological role in competition among pseudomonads or, potentially, other pyocin S3-related DNase bacteriocin producers occupying similar environments. Production of one such bacteriocin, carocin S1, by the soft rot-causing γ -proteobacterial phytopathogen *Pectobacterium carotovorum* was reported (Chuang *et al.*, 2007).

Putative novel pyocins with a tandem DNase architecture

Six predicted S1-type pyocins (*Pseudomonas mandelii* 36MFCvi1.1, *Pseudomonas* spp. 45MFCol3.1, GM33, GM50, GM79, and GM102) and four putative S3-types pyocins (*P. fluorescens* NCIMB 11674, *Pseudomonas plecoglossicida* NB2011, *P. putida* GB-1, and CSV86) have an unusual domain composition. Between the 'regular' carboxy-terminal and amino-terminal regions, they contain a similar extra segment of c. 250 amino acids, consisting of a pyocin-diagnostic domain (Pyocin_S; PF06958) followed by a pyocin S1-like cytotoxic domain (Colicin-DNase; PF12639; Fig. 1). Hence, these putative bacteriocins display a tandem DNase architecture. The extra internal nuclease domains constitute a phylogenetic cluster that is related to – but distinct from – the HNH DNase domains as present in the pyocin S1–S2–AP41 family (Fig. 3). These hybrid pyocins are tentatively designated pyocin H1 (with S1–S1 organization) and pyocin H2 (with S1–S3 organization).

These 10 atypical pyocin-like genes encode proteins with length ranged from 632 amino acids (strain NB2011) to 872 amino acids (strain CSV86) and are found in four different genomic contexts but, remarkably, their cognate immunity gene is consistently followed by a gene encoding a putative immunity-like protein with a peculiar domain configuration. The latter is composed of a carboxy-terminal Pyocin_S domain (however not connected to any cytotoxic domain) and an amino-terminal domain quite similar to pyocin S1-type immunity protein, connected by a sequence of around 265 amino acids (Fig. 1). The different genomic locations are somehow reflected in the sequence similarity of these architecturally similar

proteins. Five of them show orthology (> 80% amino acid identity for rhizosphere isolates 36MFCvi1.1, 45MFCol3.1, GM50, GM79, and GM102), but their homology to the other proteins is moderate to low (from c. 50% for strains GM33 and NCIMB 11674 to < 30% for strains CSV86, GB-1, and NB2011). Notably, this strain set encompasses not only several rhizosphere isolates (including GM33) but also strains originating from quite diverse environments: the cyanide-utilizing river mud isolate *P. fluorescens* NCIMB 11674 (Vilo *et al.*, 2012), the manganese-oxidizing freshwater isolate *P. putida* GB-1 (Wu *et al.*, 2011b), the naphthalene degrader *P. putida* CSV86 isolated from soil (Phale *et al.*, 2013), and the fish pathogen *P. plecoglossicida* NB2011 (Mao *et al.*, 2013).

Despite the considerable divergence across these strains, which seems not to be dependent on the nature of the carboxy-terminal DNase domain already present (pyocin S1- or pyocin S3-like), the three-gene synteny is conserved. Furthermore, the quite similar cluster topology of the amino-terminal immunity domains vs. the upstream-encoded immunity proteins (Figs S1 and S3), strongly hints to a functional module in which accessory toxin/immunity pairs coevolved in separate strains. The unprecedented domain architecture of such chimeric immunity protein suggests that this may be required for self-protection against the expanded cytotoxic capacity engendered by the supplemental DNase activity. If so, such dual-activity toxin might be produced as a trimeric complex with both of its immunity protomers.

tRNase pyocins

Pyocin S4-like tRNases

In addition to *P. aeruginosa* PAO1, the colicin E5-like pyocin S4 gene pair is found in about 24 currently available genomic sequences of this species, a number quite comparable to the occurrence of pyocin S3 family members. In both cases, only few of the immunity genes, although as highly conserved as the upstream toxin gene, are actually annotated. The pyocins S3 and S4 distribution profiles among *P. aeruginosa* strains show however little overlap (Table S1). Strains showing this dual pyocinogenic capacity are UCBPP-PA14, BL16, and BWHPSA027. Conversely, nearly, half of the pyocin S4-positive strains combine this feature with genes encoding either pyocin S2 (as found in strain PAO1) or pyocin S1, but no co-occurrence with pyocin AP41 is noted. Highly in contrast to the other S-type pyocin families, this tRNase toxin gene pair is rare among other pseudomonad species. Moreover, the single currently identifiable homologous system, found in *P. fluorescens* NCIMB 11674, displays only moderate amino acid similarity for the

cytotoxic domain and for the immunity protein (54% and 32% amino acid identity, respectively), and the divergent amino-terminal part of *c.* 300 amino acids is less than half the size of the equivalent pyocin S4 region (Fig. 1). A comparable level of similarity is present in a putative orphan immunity protein that is encoded in a 8-kb genomic region of unknown function, carrying some Rhs-like proteins (see Rhs elements as mediators of inter-cellular competition) and apparently inserted between pyoverdine biosynthetic gene clusters of *P. fluorescens* F113 (Redondo-Nieto *et al.*, 2013).

Pyocins carrying ColD-like tRNase domains

Quite a number of *P. aeruginosa* strains carry a gene coding for a bacteriocin with a second type of tRNase domain (Colicin_D; Pfam PF11429; Fig. S4). Their carboxy-terminal domain shows significant similarity to the cytotoxic domain of *E. coli* colicin D (Cascales *et al.*, 2007), *Klebsiella oxytoca* klebicin D (Chavan *et al.*, 2005), and *Pe. carotovorum* carocin S2 (Chan *et al.*, 2011). Colicin D cleaves anticodon loops of at least three of the four tRNA^{Arg}, in contrast to the tRNase from colicin E5 (same type as present in pyocin S4) that prefers tRNA^{Asn}, tRNA^{Asp}, tRNA^{His}, and tRNA^{Tyr} (Papadakos *et al.*, 2012). The biological activity of this novel type of *P. aeruginosa* bacteriocin, currently found in about 35 strains, has not yet been investigated. In *P. aeruginosa*, this family harbors two different subtypes (pyocins S11 and S12, Table 1) that carry similar tRNase modules and immunity proteins (*c.* 65% and 54% amino acid identity, respectively) but differ in their amino-terminal domains (Fig. S5). For pyocin S11, it closely resembles the equivalent part of the HNH DNase pyocin S2, whereas pyocin S12 shares this domain with the non-HNH DNase pyocin S3 (Fig. 1). These similarities suggest that pyocins S11 and S12 recognize receptors on susceptible cells similar to those targeted by pyocins S2 and S3, respectively. The considerable sequence divergence of the cytotoxic domains, compared with colicin D and also between pyocins S11 and S12, suggests that their tRNA specificities may differ.

The S11-type is the most abundant, occurring in about 30 strains, including *P. aeruginosa* LESB58 (Liverpool Epidemic Strain B58; Winstanley *et al.*, 2009). The multiresistant strain PA7 (Roy *et al.*, 2010) and a few other isolates (BL01, BL21, S54485, X13273) encode the S12 type of pyocin. The misleading annotation of these proteins as pyocins S2 (strain LESB58) and S3 (strain PA7) reflects a common problem with reliable automatic annotation of such modular bacteriocins in which the cytotoxic moiety occupies only a small part of the toxin.

A number of strains display dual tRNase bacteriocinogeny mediated by pyocins S4 and S11 (strains 2192, BWHPSA009, BWHPSA028, SCV20265, WC55), but none of these isolates has the capacity to additionally produce a DNase pyocin (Table S1). Combination of colicin D-like tRNase and pyocin AP41-mediated DNase activities is however found in at least two strains, either containing a LESB58 ortholog (strain BL07) or a PA7 ortholog (strain BL01). Remarkably, not only pyocin S4-type tRNase-mediated antagonistic potential is very rare in pseudomonad species other than *P. aeruginosa*. Also, the colicin D-type of tRNase is largely confined to this single species.

Pore-forming pyocins

Nearly, 20 *P. aeruginosa* strains, including UCBPP-PA14, possess highly conserved orthologs of the pyocin S5 genes of strain PAO1, making it one of the smaller group of pyocins in this species. Most of the strains also contain a pyocin system based on DNA degradation (pyocins S1, S2, or S3) and/or tRNA degradation. For the latter category, pyocin S4 co-occurrence seems to be favored as it is combined with pyocin S5 in more than half of the strains, while pyocin S12 genes are absent in the identified S5 pyocinogens (Table S1).

The inspection of pseudomonad genomes other than *P. aeruginosa* reveals additional bacteriocins with carboxy-terminal domains and cognate immunity proteins that display significant homology with the pore-forming domain and immunity protein of pyocin S5 (*c.* 50% and 30–35% amino acid identity, respectively; Fig. 1). This sequence conservation contrasts with the diversity of their amino-terminal domains which range in length from *c.* 190 residues (*P. fluorescens* 2–92) to *c.* 375 residues (*Pseudomonas* sp. GM60) and bear little sequence similarity among each other, apart from those encoded by *P. fluorescens* strains SBW25, A506, and – to a lesser extent – S12 (Fig. 1).

rRNase pyocins

Until recently, a counterpart of the 16S rRNase bacteriocins colicin E3 and cloacin DF13 was not characterized in a pseudomonad strain. A candidate gene cluster, identified in the epidemic cystic fibrosis clone CF-PA39, was shown to encode a new type of *P. aeruginosa* bacteriocin, designated pyocin S6 (Dingemans *et al.*, 2013). Pyocin S6 is nearly identical to pyocin S1, except that its carboxy-terminal domain conferring DNase activity is replaced with a colicin E3-like module, likely to mediate killing by breakdown of ribosomal RNA (Fig. 1). The pyocin S6 operon is also present in an incorrectly annotated

genomic stretch of strain PA45 (Segata *et al.*, 2013). Inspection of *Pseudomonas* genomes revealed yet another colicin E3-like gene pair, but in this case, the rRNase domain is fused to an amino-terminal domain nearly identical to the equivalent part of pyocin S2. This additional pyocin system, designated here pyocin S7 (Table 1), is harbored by two other *P. aeruginosa* strains, JD312 (genes not annotated; Dettman *et al.*, 2013) and BWHPA018 (Fig. 1). The corresponding immunity proteins of pyocin S6 and pyocin S7 are identical.

Compared with the other pyocin families, the ribosomal RNA-targeting system appears to have by far the narrowest distribution among *P. aeruginosa* isolates. This is in stark contrast with the predicted abundance of this type of toxin family in other pseudomonads. Using the amino acid sequences of the colicin E3 catalytic domain and of the cognate immunity protein as queries, a large number of gene tandems (*c.* 60) potentially encoding several novel rRNase bacteriocin/immunity pairs emerged. Phylogenetic analysis of the cytotoxicity-mediating domain sequences (covering about 95 residues) of a representative subset highlights their pronounced and species-independent diversity, although two main branches can be distinguished (Fig. 4). This dichotomy is even more pronounced for the respective immunity proteins (Fig. S6). In the subset, most related to the colicin E3 immunity protein the diagnostic Pfam PF03513 domain

is detected. This is not the case for the subset with somewhat shorter immunity proteins, including those of *P. aeruginosa* pyocins S6 and S7.

Some strains carry two rRNase bacteriocin operons at unlinked genomic locations. These pairs of cytotoxic domains and immunity proteins exhibit significant sequence relatedness for some strains (clustering in one main branch), but others exhibit only remote sequence similarity (present in different main branches). The pronounced phylogenetic dichotomy suggests that the latter systems may not share a common ancestor. The respective toxin pairs of a particular strain also carry rather divergent amino-terminal domains with amino acid identities ranging from *c.* 60% for *P. chlororaphis* O6 to only 35% for *P. chlororaphis* subsp. *aureofaciens* 30–84. The majority of the pyocin S6-related bacteriocins consist of about 400 amino acids, but the size varies between 642 amino acids for *P. aeruginosa* strains with the pyocin S2-type domain and only 280 amino acids for *Pseudomonas* sp. Ag1. Similar to the amino-terminal domain sharing between pyocins S6 and S1, and between pyocins S7 and S2, three *P. chlororaphis* strains encode a protein with a 90% identical amino-terminal domain (Fig. 1) but linked to a different cytotoxic domain. *Pseudomonas chlororaphis* O6 [Pchl O6 (b)] carries a rRNase domain, whereas *P. chlororaphis* subsp. *aurantiaca* PB-St2 and *P. chlororaphis* subsp. *aureofaciens* 30–84 bear a HNH domain [Pchl

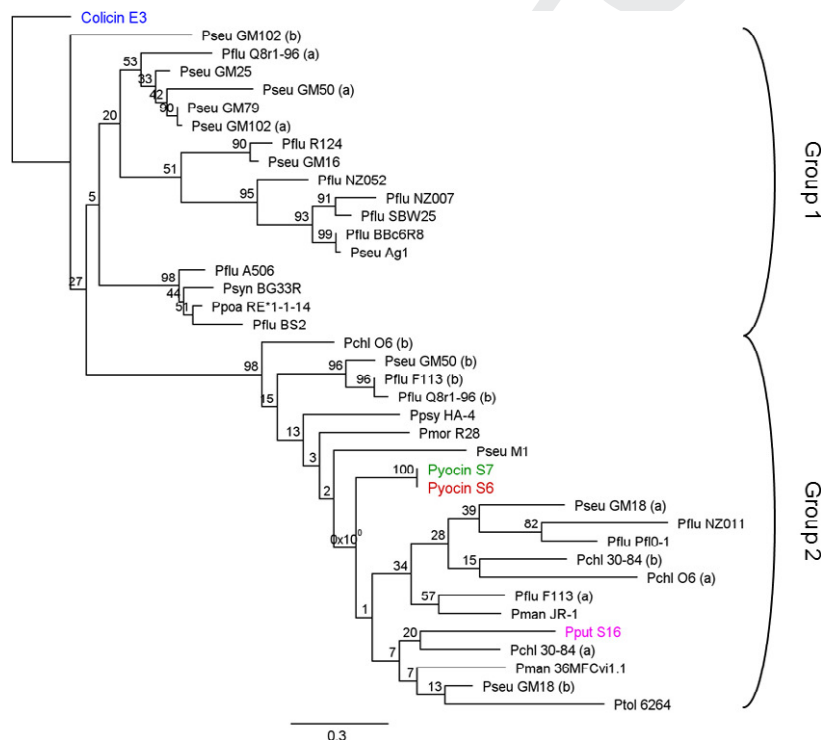


Fig. 4. Phylogenetic analysis of the cytotoxic domains in representative pseudomonad bacteriocins of the novel pyocin S6 family. Based on alignment of the carboxy-terminal domains, a ML phylogenetic tree was constructed (rooted with the *Escherichia coli* colicin E3 rRNase domain; blue). Two predicted rRNase bacteriocins in a particular strain are discriminated by extensions (a) and (b). The *Pseudomonas aeruginosa* members (red and green) and an Rhs protein (pink) are highlighted. The two main groups of rRNase cytotoxic domains are indicated (groups 1 and 2). Abbreviations used for species names: Pmor, *Pseudomonas moraviensis*; Ppoa, *Pseudomonas poae* (others as in the legend of Figs 1 and 3). The scale bar represents 0.3 substitutions per site. Bootstrap values (percentage of 100 replicates) are shown at the branches.

PB-St2 (b)]. For the latter two proteins, the amino-terminal domains are better conserved than the DNase modules (only c. 60% id).

Also, for S6-type pyocins, expanded immunity gene clusters have been assembled in some strains. For instance, *Pseudomonas tolaasii* 6264 and *Pseudomonas* sp. GM18 (a cluster, Fig. 4) carry, just downstream of their self-immunity gene, respectively, one and two quite different immunity genes (Fig. 1). The quite low overall sequence conservation between the pyocin S6 immunity proteins, along with the deviating codon usage and their small size (75–95 amino acids), hampers their identification and genomic annotation. Manual inspection on the other hand is facilitated by the consistent tight operon-like linkage with the cognate rRNase gene, similar to the other nucleic acid-degrading systems.

The rRNase-type bacteriocinogenic capacity seems to be common in populations of nonpathogenic pseudomonads isolated from soil and plant environments, including several biocontrol-active pseudomonads: *P. fluorescens* strains A506, F113, and SBW25, *P. chlororaphis* strains 30–84 and O6, *Pseudomonas brassicacearum* Q8r1-96, *P. synxantha* BG33R, and *Pseudomonas poae* RE*1-1-14 (Fig. 1; Loper *et al.*, 2012; Müller *et al.*, 2013; Redondo-Nieto *et al.*, 2013). This property is also present in several poplar rhizosphere and endosphere isolates (Brown *et al.*, 2012): five strains included in the phylogenetic comparison (GM18, GM25, GM50, GM79, GM102), and additionally, five strains not shown in the comparative analysis (GM17, GM21, GM30, GM41, GM48). Thus, in this sample, about half of the 21 strains sequenced is potentially able to produce one or two bacteriocins with this killing activity. Six of these strains (underlined) also display HNH-type DNA-targeting bacteriocinogenic potential, suggesting a relatively wide distribution and probable significant ecological role of such complementary nuclease-dependent antagonism in plant root environments. Also, one middle-sized Rhs protein (417 amino acids) equipped with a rRNase module and cognate immunity protein was identified in the nicotine-degrading soil isolate *P. putida* S16 (Yu *et al.*, 2011), indicating that such nuclease module can also be shared by different types of bacteriotoxins (Fig. 1).

Remarkably, the prominent phyllosphere inhabitant and plant pathogen *P. syringae* seems not to make use of this type of antagonistic proteins. Given the observation that pyocin S6 is also very rare among *P. aeruginosa* isolates, its presence may provide little competitive advantage during colonization or infection of their eukaryotic hosts. Alternatively, it may actually represent a recent acquisition of soilborne pseudomonad origin. It should be pointed out that a considerable number of *P. aeruginosa* isolates lacking the pyocin S6 toxin gene, do carry a close

homolog of the pyocin S6 immunity gene. For instance, in CF-isolate C7447m (Yin *et al.*, 2013), this orphan is located downstream of the pyocin S2 gene pair of this strain. It is preceded by a small unannotated ORF that would encode a pyocin S6-type of toxic domain, which is reminiscent of the minimal toxin-CT/immunity modules (Poole *et al.*, 2011). It was suggested that such gene pairs might contribute to immunity but, by retaining the toxin warhead sequence, could also serve as a reservoir for future assembly of novel antagonistic capacities.

Lipid II-degrading bacteriocins of the pyocin M family

Screening of pseudomonad genomes using the pyocin M amino acid sequences from PaeM (pyocin M1), syringacin M (pyocin M2), and PflM (pyocin M3) as queries revealed several candidate lipid II-targeting bacteriocins (Fig. 1, Table S1). An identical ortholog of PaeM is found in a limited number of *P. aeruginosa* isolates (e.g. strains 6077, 39016, BL08, BL14, BL17, E2, JJ692, MH27, U2504), which reflects the distribution of the 80-kb genomic island carrying the corresponding gene (*exa13*) in addition to the virulence factor *exoU* (Kulasekara *et al.*, 2006). In some other *P. aeruginosa* strains (e.g. BL01, BL03, BWHPA008, JD332), a distantly related *colM*-like gene was identified (encoding pyocin M4, Table 1), adjacent to the conserved *arc* operon for anaerobic arginine catabolism (Verhoogt *et al.*, 1992). Genes encoding close syringacin M homologs have integrated at different locations in *P. syringae* pathovar genomes. Most of the proteins are composed of nearly identical killing domains (> 95% amino acid identity; Fig. 5) and very similar translocation and receptor domains (> 90% AA-identity). However, some of these strains (e.g. *P. syringae* pv. *mors-prunorum* M302280 and *P. syringae* pv. *theae* ICMP3923) carry genes coding for a second, only distantly related and less abundant colicin M-like protein, a variant of which is also found in *P. syringae* pv. *aceris* M302273 (Fig. S7).

Considerable diversification of this type of bacteriocinogenic potential is apparent among the few other strains identified, mostly nonpathogenic isolates from plants. Their enzymatic domains share < 45% amino acid identity with syringacin M or PaeM (Fig. 5). The amino-terminal domains are of three different types showing no significant sequence similarities among them. They are either (distantly) related to PaeM (strains GM21, 6264, BG33R, DF41, NFM4421) or to syringacin M (all *P. syringae* strains), with a third group consisting of putative M-type pyocins from *P. fluorescens* HK44 (Chauhan *et al.*, 2011) and *Pseudomonas* sp. GM33, GM49, GM55, and NZ011 (Fig. S7). The amino-terminal region of

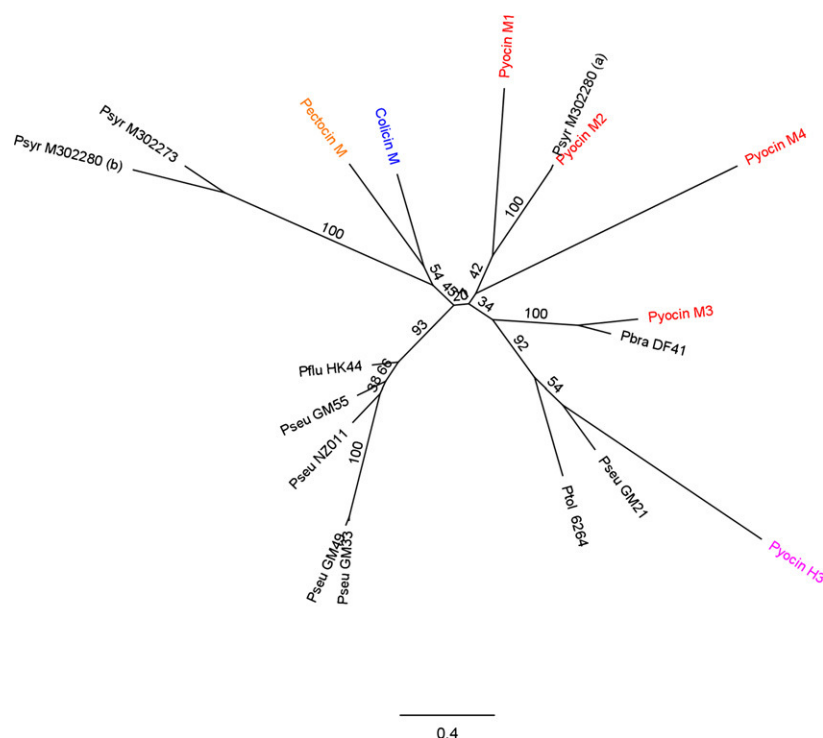


Fig. 5. Phylogenetic analysis of cytotoxic domains present in representative colicin M-like pseudomonad bacteriocins (red), *Escherichia coli* colicin M (blue) and *Pectobacterium carotovorum* pectocin M (orange). In pyocin H3 (pink), the peptidoglycan-degrading pyocin M domain is linked to an extra pore-forming colicin N-like domain at its carboxy-terminus. Abbreviations used for *Pseudomonas* species names can be retrieved in the legend of Fig. 1. The scale bar represents 0.4 substitutions per site. Bootstrap values (percentage of 100 replicates) are shown at the branches.

P. aeruginosa pyocin M4 represents a fourth type. However, such lack of discernable sequence similarity does not exclude a similar 3D architecture. In this bacteriocin family, a very similar killing domain can be combined with sequence-unrelated moieties, as exemplified by colicin M, syringacin M and pectocin M from *Pe. carotovorum* (Fig. 5). For the latter protein, it will also be of interest to see how the amino-terminal domain, which shows striking similarity to plant [2Fe–2S] ferredoxins (Grinter *et al.*, 2012a), is folded and integrated with the enzymatic domain.

The domain flexibility of these bacteriocins is further evident from a protein, encoded by the nematocidal strain *P. synxantha* BG33R that displays an unprecedented domain combination. This putative colicin M-like bacteriocin is fused at its carboxy-terminus with an extra domain that bears similarity (*c.* 35% amino acid identity) to pore-forming modules as found in colicin N (Vetter *et al.*, 1998). The presence of a downstream convergent immunity gene of the same category (GenBank Accession Number EIK72461) suggests that this may represent a toxin capable of interfering with both peptidoglycan assembly and cytoplasmic membrane integrity. This hybrid pyocin carrying two functionally unrelated inhibitory modules is designated pyocin H3 (Fig. 1).

In a number of strains, the M-type pyocins are encoded by cargo genes on prophages. This is the case for the related prophages that occupy the *trpE–trpG* intergenic regions in

P. syringae pv. *tomato* DC3000 (pyocin M2) and *P. syringae* pv. *syringae* 642 (pyocin M2 ortholog), as well for the same prophage inserted between *mutS* and *cinA* in *P. fluorescens* Q8r1-96 (pyocin M3) and *P. brassicacearum* subsp. *brassicacearum* NF4421 (pyocin M3 ortholog). However, acquisition of the gene encoding a pyocin M3 homolog (65% amino acid identity) in another *P. brassicacearum* strain (DF41) apparently occurred independently of this type of mobile element.

Tailocins: phage tail-like bacteriocins

Phage tail-like bacteriocins represent a second class of antibacterial defence weapons, present in many proteobacterial genera. In *Pseudomonas*, two major classes of phage tail-like particles have been described, viz. R-type and F-type pyocins (Michel-Briand & Baysse, 2002). The more general term ‘tailocins’ has been proposed to mark the widespread occurrence of these antibacterials (Gill & Young, 2011).

R-type pyocins

R-type pyocins have mainly been studied in *P. aeruginosa* and display activity against other strains of this species. Multiple strains producing R pyocins have been described and they are classified according to their target spectrum. Currently, five subgroups have been characterized, named

R1–R5 (Michel-Briand & Baysse, 2002; Leiman & Shneider, 2012). Some additional R-type pyocins, not assigned to one of these groups, include C9 (Higerd *et al.*, 1967), pyocin 21 (Govan, 1974a), and 430c (Govan, 1974b).

Structure and mode of action of R-type pyocins

R-type pyocins closely resemble T-even bacteriophage tails and appear as rigid and non-flexuous particles. Electron microscopy study suggests that they are built from a double hollow cylinder, consisting of a rigid inner core and a contractile outer sheath, 1200 Å long and 150 Å in outer diameter. The surface of the extended sheath contains different sets of striations, originating from the helically structured six subunit-containing annuli. A baseplate is attached to the sheath and serves as a docking point for the six tail fibers functioning as an anchor for attachment to target cells (Ishii *et al.*, 1965; Higerd *et al.*, 1969; Takeda & Kageyama, 1975). The specific antigens defining target strain specificity are located at the distal portion of the fibers and categorize the pyocins in one of the five classes (R1–R5; Ohsumi *et al.*, 1980; Kumazaki & Ishii, 1982).

Upon contact, the sheath contracts and the pyocin core is inserted into the cell envelope (Higerd *et al.*, 1969; Govan, 1974a; Shinomiya *et al.*, 1975; Uratani, 1982). The pyocin forms a channel through the inner membrane that causes depolarization of the membrane (Uratani & Hoshino, 1984) and effects arrest of protein and nucleic acid synthesis (Kaziro & Tanaka, 1965; Ohsumi *et al.*, 1980), ultimately resulting in cell death. This killing is very efficient as attachment of only a single particle may result in cell death (Kageyama *et al.*, 1964).

Receptor of R-type pyocins

The receptor of R-pyocin tail fibers is mainly constituted of lipopolysaccharide. It was observed that upon addition of purified lipopolysaccharide, survival of cells to such pyocin was higher (Ikeda & Egami, 1969; Govan, 1974a, b; Meadow & Wells, 1978; Kumazaki *et al.*, 1982). More specifically, the carbohydrate moieties of the lipopolysaccharide are part of the receptor sites. Using defined lipopolysaccharide mutants, a L-rhamnose residue and two distinct D-glucose residues in the lipopolysaccharide outer core were found to participate in the receptor site of pyocin R1, R2, and R5, respectively (Köhler *et al.*, 2010). For pyocin R3, the absence of a terminal D-glucose residue in the lipopolysaccharide core confers resistance to R-pyocin killing (Kocincova & Lam, 2013).

Interestingly, R pyocins from *P. aeruginosa* also target strains of other bacterial genera, such as *Campylobacter* sp. (Blackwell *et al.*, 1982), *Haemophilus ducreyi* (Cam-

pagnari *et al.*, 1994), *Haemophilus influenzae* (Phillips *et al.*, 1990), *Neisseria gonorrhoeae* (Morse *et al.*, 1976; Blackwell *et al.*, 1979), and *Neisseria meningitidis* (Blackwell & Law, 1981), suggesting that these bacteria share common lipopolysaccharide receptors with *P. aeruginosa* (Connelly & Allen, 1983; Filiatrault *et al.*, 2001).

Genetic determinants of R-type pyocins

The genetic locus of pyocin R2 was taken into focus in *P. aeruginosa* PAO1 (Nakayama *et al.*, 2000). Located between *trpE* and *trpG*, the pyocin R2 cluster is followed downstream by a pyocin F2 locus, separated from the latter by a lysis gene cassette (Fig. 6). The 16-ORF gene cluster (*prf10*–*prf23*, encoding PA0614–PA0627) displays similarities to phage P2, 186, and ΦCTX. Moreover, the gene order of the P2 homologs is quite conserved, with minimal rearrangements. This allows assignment of functions to these different ORFs as being involved in the assembly and the formation of the core and sheath, the tail fibers, and the baseplate. The R2 locus does not bear genes involved in head formation, replication and integration, despite its common ancestry with P2 phages, suggesting an independent evolution. Hence, R-type pyocins should be considered evolutionarily highly specialized phage tails, rather than defective phages (Nakayama *et al.*, 2000). The common ancestry of R-type pyocins with bacteriophages is underlined by immunological cross-reactivity with bacteriophage PS17 (Shinomiya, 1984; Shinomiya & Ina, 1989).

Overall, the DNA sequences of the R-pyocin genomic regions are well conserved. The highest sequence divergence is noted for the tail fiber genes (Fig. 7). In R-pyocin-mediated killing, the tail fiber protein TfpH (Prf15/PA0620 in pyocin R2 of strain PAO1) is a major determinant for specific recognition of a target strain. Reflecting this diversifying role, the gene pair encoding TfpH and its cognate assembly chaperone (Prf16/PA0621 in pyocin R2 of strain PAO1) show the least conservation among pyocin R genes. At this point, five subtypes (R1 through R5) of Prf15 are known (Michel-Briand & Baysse, 2002; Williams *et al.*, 2008). Although conferring different host ranges, the amino acid sequences of Prf15-R3 and Prf15-R4 are nearly identical to Prf15-R2 (strain PAO1), but Prf15-R1 (for instance of strain LESB58; Köhler *et al.*, 2010) and Prf15-R5 (for instance of strain PABL056; Ozer *et al.*, 2012) have quite different carboxy-terminal sequences. When considering these three main biochemical types for amino acid sequence-based comparison, a prevalence of the R2/R3/R4-types (26 strains) and R5-type (21 strains) compared with the less frequently occurring R1-type (12 strains) becomes apparent (Table S1). In *P. aeruginosa* PA7, a distinct putative tail fiber protein is

encoded that is much smaller (*c.* 400 amino acids) and lacks significant amino acid similarity apart from a conserved amino-terminal domain of unknown function (DUF3751; Pfam PF12571; Fig. 6). Currently, no orthologs of this additional type (designated here as R6) and the corresponding chaperone are known in other strains.

R-type pyocin retargeting and potential applications

Taking into account that R-type pyocins display very specific and potent bactericidal activities, these antibacterials

may be considered for use as chemotherapeutic agents. Moreover, R- and F-pyocins are protease-resistant, which is not the case for S pyocins. The efficacy of R-type pyocins has been validated in *P. aeruginosa*-infected mice by intraperitoneal and intravenous administration (Merrikin & Terry, 1972; Haas *et al.*, 1974; Scholl & Martin, 2008; Ritchie *et al.*, 2011). The rather narrow antibacterial spectrum of R-type pyocins is a main disadvantage however and may compromise their clinical usefulness in the end.

As mentioned in section Genetic determinants of R-type pyocins, target strain specificity of R pyocins is mainly determined by the tail fibers, encoded by *prf15*.

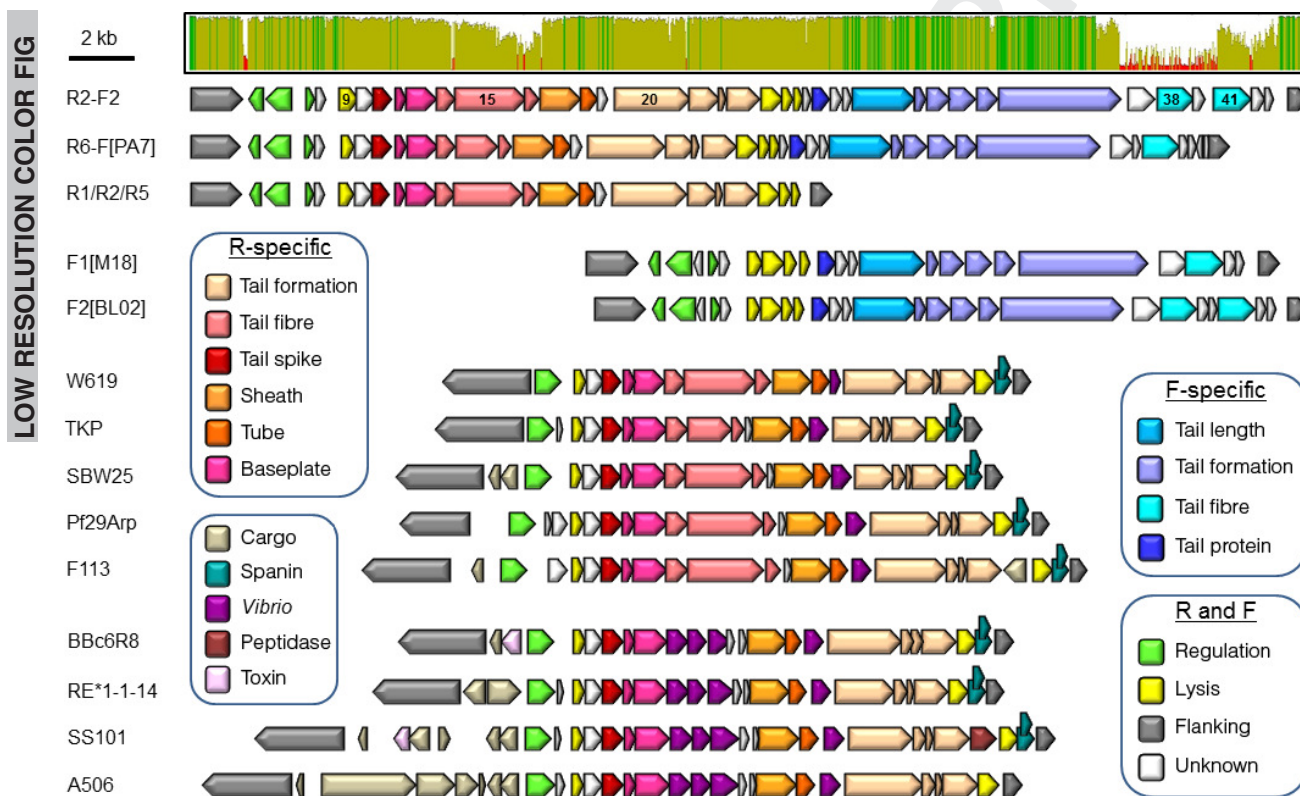


Fig. 6. Synteny of individual and fused R- and F-type pyocin gene clusters in pseudomonads. The gene organizations are compared with the R2/F2 genes of *Pseudomonas aeruginosa* PAO1. Homologous genes are represented in the same color, with color coding based on the functions assigned to the genes in strain PAO1. In addition, putative pyocin and cargo genes absent from the *P. aeruginosa* systems are highlighted in distinct colors. The upper box visualizes the extent of nucleotide sequence conservation (fully conserved, bright green; poorly conserved, red) among aligned dual R/F clusters of the eight different combinations types discussed in the text. The more divergent combined R/F cluster of strain *P. aeruginosa* PA7 is shown for comparison. In addition to genes discussed in the text (*prf9*, *prf20*), the tail fiber protein-encoding genes of strain PAO1 are numbered for the R pyocin (*prf15*) and F pyocin (*prf38*, *prf41*; Nakayama *et al.*, 2000). As a representative for the highly similar gene clusters encoding R1- through R5-type pyocins, the genomic region of the mono-R strain LESB58 is shown. The genetic backbone of a F1- and F2-pyocinogenic strain is illustrated with the coding regions of strains M18 and BL02. Putative pyocin R-like gene organizations of type R-a are shown for *Pseudomonas fluorescens* strains F113, Pf29Arp and SBW25, *Pseudomonas putida* W619, and *Pseudomonas* sp. TKP (not included similar clusters: *P. fluorescens* Q2-87; *Pseudomonas* spp. Ag1, GM60, and GM67). The R-b type of organization is illustrated for *P. fluorescens* A506, BBc6R8, and SS101 and for *Pseudomonas poae* RE*1-1-14 (not included similar clusters: *Pseudomonas* sp. CF150 (99% identity to the A506 sequence) and *Pseudomonas* sp. FH4). The pyocin genes are all located in the *trpE*–*trpG* intergenic regions of *P. aeruginosa* strains and between *mutS* and *cinA* of other pseudomonads, with the respective regulatory genes proximal to *trpE* or *mutS*. The scale bar represents a genomic region of 2 kb.

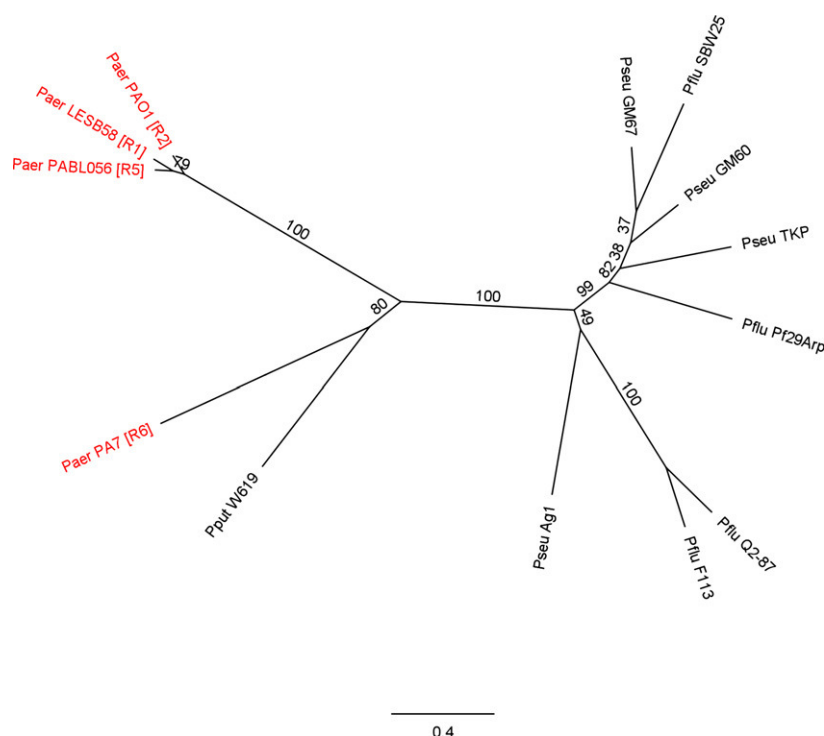


Fig. 7. Phylogenetic analysis of tail fiber proteins encoded by R-type pyocin genes of *Pseudomonas aeruginosa* and other pseudomonads. The major R-types are represented by sequences from *P. aeruginosa* strains LESB58 (R1), PAO1 (R2; nearly identical to R3 and R4), PABL056 (R5), and PA7 (new type R6), all colored red. Abbreviations used for species names can be retrieved in the legend of Fig. 1. The scale bar represents 0.4 substitutions per site. Bootstrap values (percentage of 100 replicates) are shown at the branches.

Interestingly, the exchange of *prf15* of pyocin R2 with the corresponding tail fiber genes of other R-type pyocins results in functional bacteriocins with the target spectrum of the tail fiber ‘donor’ (Williams *et al.*, 2008). Taking this idea further, the antibacterial spectrum of R2 can also be diversified by replacement with the tail fiber of a *P. aeruginosa*-specific bacteriophage, as demonstrated for PS17. Engineered pyocins targeting *E. coli* and *Yersinia pestis* strains were created by fusing the conserved R2 amino-terminal region-encoding portion of *prf15* to the variable carboxy-terminal parts of the phage tail fiber genes. To ensure proper assembly and optimal activity of the engineered pyocins, coexpression of the cognate chaperone protein Prf16 is recommended (Williams *et al.*, 2008). Several artificial pyocins carrying chimeric tail fiber genes were engineered. Targets include a number of food-borne pathogens, such as *E. coli* O157:H7 (Scholl *et al.*, 2009; Ritchie *et al.*, 2011) and *E. coli* O104:H4 (Scholl *et al.*, 2012). Useful candidate bacteriophage tail spike proteins for introduction in the R-type pyocins may be selected after genome screening and identification of lysogenic bacteriophages in the corresponding bacterial genomes of interest (Scholl *et al.*, 2012).

An additional advantage of these engineered highly specific bacteriocin particles is that their potent activity does not induce Shiga toxin (Stx) production (Scholl *et al.*, 2009). There is clinical evidence that antibiotic therapies

of Stx-producing *E. coli* may lead to an enhanced risk of hemolytic-uremic syndrome (Wong *et al.*, 2000). The potential usefulness of these engineered pyocins against *E. coli* O157:H7 has been demonstrated in a rabbit model of diarrheal disease by orogastric administration (Ritchie *et al.*, 2011) and in the decontamination of beef surfaces (Scholl *et al.*, 2009). This way, R-type pyocins may serve as an engineering platform for the production of different, highly specific antimicrobials targeting virtually any pathogen of interest without disrupting the normal host microbiota.

F-type pyocins

A second type of *P. aeruginosa* phage tail-like bacteriocins are the F pyocins. These particles do not exhibit the typical sheath-core structure of R pyocins. Several F-type pyocins have been reported: pyocin 28 (Takeya *et al.*, 1967), 430f (Govan, 1974b), F1 and F2 (Kuroda & Kageyama, 1979) and F3 (Kuroda & Kageyama, 1981). Their production is often accompanied by a coexpressed R-type pyocin or bacteriophage (Govan, 1974b; Kuroda & Kageyama, 1979).

F-pyocin structure

Electron microscopy study demonstrated that F-type pyocins appear as flexuous, noncontractile rods, with an

estimated length of 106 nm and width of 10 nm. Contrary to the pyocin length that may not be uniform, the width is fixed with regular striations demarcating the individual rings or annuli, 23 in total, constituted by a 19.5-kDa protein as main subunit protein. One end of the F-type pyocin is square, while the other tapers to a point from which a fiber complex originates, allowing the binding to a receptor cell. The latter structure is composed of several long and short filaments, with an estimated length of 43 nm (Takeya *et al.*, 1969; Kuroda & Kageyama, 1979; Kuroda *et al.*, 1979; Kuroda & Kageyama, 1981). These fibers are the main determinant of specificity and, consequently, are antigen specific (Kuroda & Kageyama, 1981; Kuroda & Kagiya, 1983). Kinetic analysis of killing suggests that F pyocins act by a single-hit process, similar to R-type pyocins (Kuroda & Kageyama, 1979; Kuroda *et al.*, 1979).

Genetic organization of F-type pyocins

The genetic determinants of pyocin F2 have also been studied in detail in *P. aeruginosa* PAO1 (Nakayama *et al.*, 2000). Genetic analysis of this pyocin revealed a 16-ORF gene cluster (*prf28–prf43*, encoding PA0632–PA0648), downstream of a pyocin R2 gene cluster and separated from the latter by a lysis gene cassette (Fig. 6). Genetic similarities and synteny with λ phage suggest that several ORFs are involved in rod formation, guaranteeing overall integrity and proper length of the tail structure. Additional downstream-located genes are involved in the formation of the fiber-like filaments. They are organized as two similar three-gene cassettes, both of which are thought to be functional. *Pseudomonas aeruginosa* PML14 equally produces pyocin F2 and harbors a similar gene cluster with minimal changes but is not accompanied by a R-pyocin gene cluster (Nakayama *et al.*, 2000; Michel-Briand & Baysse, 2002). The pyocin F1 cluster lacks such seemingly duplicated 3'-region (Nakayama *et al.*, 2000) and this is the case as well for all other non-F2-type pyocin clusters identified in *P. aeruginosa* genomes (see Genomic backbones of pseudomonad tailocins). Despite the apparent common ancestry with phage λ , F-type pyocins should also be regarded as evolutionarily highly specialized phage tails, similar to R-type pyocins (Nakayama *et al.*, 2000). Analogous to what is observed for R-type pyocins and related phages, anti-F sera may neutralize phages as well, such as KF1 (Kuroda & Kagiya, 1983; Kuroda *et al.*, 1983).

The highest sequence divergence among F-type pyocins is displayed by the tail fiber proteins (Fig. 6), as equally observed for the R-type pyocin fibers. The same trend is apparent for the two tail fibers within the F2 module of PAO1 [Prf38 (PA0643) and Prf41 (PA0646) (Nakayama

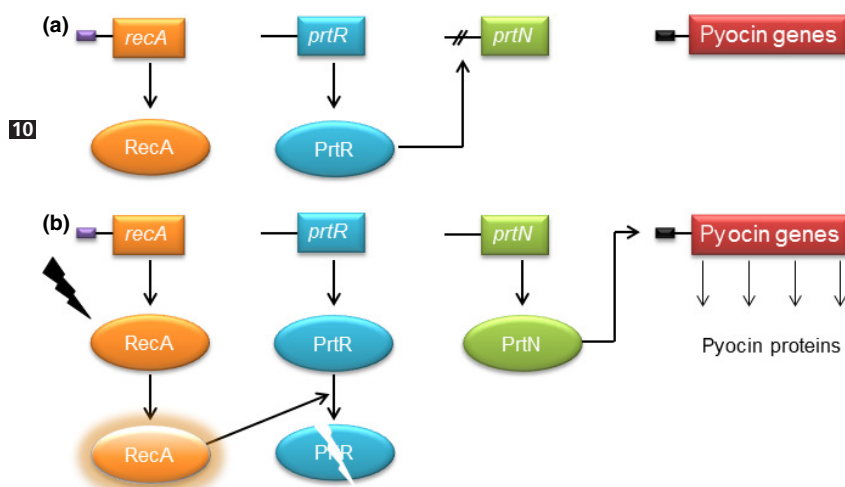
et al., 2000)]. The amino acid sequence of the pyocin F3 tail fiber protein (strain PAF41, also producing pyocin AP41) is not known (Kuroda & Kagiya, 1983). To this extent, homologs of Prf15 (*c.* 700 amino acids) and of Prf38/Prf41 (ranging in length from *c.* 350 to 460 residues) are instrumental for sequence-based differentiation of R- and F-type pyocins, respectively.

Phylogenetic analysis of predicted tail fiber proteins encoded by the F-pyocin regions of *P. aeruginosa* strains indicates the existence of additional (sub)types (Fig. S8). These protomers share a conserved amino-terminal region of about 140 amino acids, but the remaining part (ranging in length from 210 to 340 residues) has diverged strongly. The PA14 protein is clearly related to the F2-type tail fiber protein Prf41 (89% amino acid identity) and the M18 protein is most similar to the F1-type protein Prf41 (66%). Two additional types represented by the more distant homologs of strain PA7 and strain DK2 cannot be particularly linked to one of the known F-types. As the tail fiber proteins are major determinants of receptor-recognition specificity, this suggests that the PA7- and DK2-encoded pyocins adopt the general F-type tailocin architecture but likely bind to receptors different from those recognized on cells susceptible to either pyocins F1, F2, or F3. About half of the 61 F-type pyocin clusters inspected belong to the F2 type, followed in number by the DK2 type (12 orthologs). The F1 type appears much less abundant (six strains), comparable in frequency of occurrence to the M18 and PA14 proteins (Table S1).

Regulation of pyocin expression in *P. aeruginosa*

The expression of R-type, F-type, and S-type pyocins is regulated by a common mechanism in *P. aeruginosa*. Regulatory sequences called P-boxes, often presented in repeats, are located in noncoding upstream regions of the pyocin genes and clusters, and serve as a binding site for the PrtN activator (Matsui *et al.*, 1993). These P-box elements share no similarity with the SOS boxes of the colicin genes from *E. coli*, and hence, the LexA repressor is not involved. Similar to colicins, however, pyocin production was found inducible upon DNA-damaging treatment by UV light or mitomycin C, and appeared dependent on *recA* (Sano & Kageyama, 1993; Sano *et al.*, 1993b). Under stress conditions, an activated RecA will cleave the repressor protein PrtR. This will cause liberation of the promoter region of *prtN*, and hence, the expression of this gene. PrtN activator is able to bind the P-boxes which will ultimately initiate pyocin expression (Fig. 8). *prtR* and *prtN* are both small proteins encoded by genes located upstream of the R2/F2 locus in *P. aeruginosa*

Fig. 8. Model of pyocin regulation in *Pseudomonas aeruginosa*. (a) In noninducing conditions, expression of pyocin genes is repressed indirectly by PrtR. (b) After DNA-damaging treatment (thunderbolt), RecA is activated (glowing and discolored) and activates autocleavage of PrtR. Consequently, PrtR can no longer inhibit transcription of *prtN*, encoding the pyocin transcriptional activator PrtN. SOS boxes and P boxes are represented by small purple and black boxes respectively. Agents causing DNA damage include UV-light, mitomycin C, and nitrogen oxide.



PAO1, in opposite direction (Fig. 6). PrtR shares remarkable similarities with the *cI* repressor from phage Φ 80 (Matsui *et al.*, 1993). Together with *prtN*, PrtR also inhibits expression of *ptrB*. The latter gene encodes a small protein that is involved in repression of *exsA*, a master activator of the type III secretion system. *PtrB* is not involved in pyocin expression, however (Wu & Jin, 2005). This P-box regulatory system has been detected in several *P. aeruginosa* pyocins, viz. S1, S2, AP41, R2, and F2 (Shinomiya *et al.*, 1983; Sano & Kageyama, 1987; Matsui *et al.*, 1993). Pyocin S5 expression on the contrary seems not to be subjected to this regulatory scheme as there are no P-box elements in its 5' region (Parret & De Mot, 2000). Chang *et al.* (2005) found that H_2O_2 -induced oxidative stress upregulates transcription of pyocin S5. In support of this, chromatin immunoprecipitation of the region upstream of the pyocin S5 immunity gene showed it to be bound by OxyR, a H_2O_2 -responsive transactivator (Wei *et al.*, 2012). Upon treatment with the antibiotic ciprofloxacin, the R2/F2 locus displays a strong upregulation (Brazas & Hancock, 2005), contrary to treatments with ceftazidime (Blazquez *et al.*, 2006). Recently it was found that reactive oxygen species released by neutrophils, H_2O_2 stress and ciprofloxacin treatment cause their effects in a second way, via upregulation of the PrtR mRNA level. PrtR overexpression represses the endogenous *prtR* promoter activity, suggesting an autorepressive mechanism (Sun *et al.*, 2014). Similar to the pyocins of PAO1, PaeM production from *P. aeruginosa* JJ692 is upregulated upon treatment with ciprofloxacin (Barreteau *et al.*, 2012).

Several other studies hinted to additional environmental factors influencing pyocin expression, mostly based on results obtained in model strain *P. aeruginosa* PAO1. The exact role of these responses and a possible connection with the RecA/PrtR/PrtN system remain poorly under-

stood to date. Transcriptomic and phenotypic analyses indicated that pyocin production is enhanced when *P. aeruginosa* is growing in a biofilm, both under aerobic and anaerobic conditions. In addition, co-culture with a pyocin-sensitive isolate indicates that this expression is of major importance in population dynamics (Waite & Curtis, 2009). Similarly, lowered oxygen concentrations, mimicking growth conditions of *P. aeruginosa* in a cystic fibrosis lung, upregulated pyocin S2 expression (Alvarez-Ortega & Harwood, 2007). Extracytoplasmic function sigma factor PA4896 controls the expression of pyocins R2, F2, and S5, but not S2. Dependence on a surface signaling system may be rationalized by cells trying to acquire siderophores from other bacteria simultaneously trying to kill them (Llamas *et al.*, 2008). Under denitrifying conditions, *P. aeruginosa* PAO1 forms nitrogen oxide as an intermediate. The latter induces the SOS response, activating pyocin expression, and R pyocin proteins were found to accumulate in shedded membrane vesicles (Toyofuku *et al.*, 2013). Also, F pyocin protomers, as well as pyocins S2 and S5, have been identified in *P. aeruginosa* PAO1 membrane vesicles (Choi *et al.*, 2011). These vesicles play a role as vehicles mediating interbacterial competition and may also contain murein hydrolases and antibacterial quinolones (Tashiro *et al.*, 2012).

Genomic backbones of pseudomonad tailocins

In silico analysis of tailocins in *P. aeruginosa*

The *trpE*–*trpGDC* intergenic sequence represents a hot spot for integration of the R- and F-type of pyocins in *P. aeruginosa* (Nakayama *et al.*, 2000). By PCR scanning, strains were identified in which the prototypical organization of *P. aeruginosa*, with an apparent fusion of R2-F2 type of pyocin genes, was conserved but also other

configurations were proposed, in which either an R- or F-type or both types were absent. Current full and draft genomic sequence data disclose a more comprehensive picture of the particular gene organization in this region. Inspection of the fully sequenced *trpE-trpG* genomic regions of 91 *P. aeruginosa* strains showed a quite similar number of 'mono-tailocin' strains, pyocinogenic for either R (29) or F (31), as compared to 'duo-tailocin' strains, pyocinogenic for both R and F (30). A fourth type of *trpE-trpG* intergenic organization is found in the multi-drug-resistant blood isolate VRFPA06, in which a 30.3-kb DNA sequence with several coding regions of unknown function is positioned between the end of a pyocin R2 cluster and the *trpG* gene (Murugan *et al.*, 2014). The presence of a bacteriophage P4-like integrase gene flanking the R-pyocin lysis cassette genes suggests a mobile nature for this inserted DNA region (Kung *et al.*, 2010).

The different evolutionary relatedness of R- and F-type pyocins, to phage P2 and phage λ , respectively (Nakayama *et al.*, 2000), suggests that the various types of co-integrated R/F clusters were assembled from separate pre-existing R- and F-type pyocins. In the various cluster combinations, production of both types of toxic particles by a particular strain relies on a shared conserved regulatory system to trigger expression and on a common lysis cassette for their release from producer cells (Fig. 6). Such dual R/F pyocin producers are not rare. The R2/F2 co-integrated pyocin region of strain PAO1, studied as a model system for both pyocin types, occurs in at least 10 other strains. In addition, alternative combinations of different R/F hybrid pairs exist, although occurring less frequently (Table S1). In the two strains encoding an F-PA7 ortholog, the genes are clustered with an R2 pyocin region (strain BL04) or with the PA7-specific R6 genes. Other variations on the theme of hybrid pyocin assemblages are the tandems R2/F-PA14 (strains PA14, BL17 and BWHPA028) and R2/F-M18 (strain BWHPA026) in R2-pyocinogenic isolates, and the paired clusters R1/F2 (strains BL18, BL25, MTB-1, and VRFPA07) and R1/F1 (strains PAK, CF5, and MA8.1) in R1-pyocinogenic strains. Apparently, also F1- and R5-types are compatible as such co-integrated clusters occupy the *trpE-trpG* intergenic regions of strains BL21, SS4485, and X13273.

***In silico* analysis of tailocins in other *Pseudomonas* species**

Production of a phage-like bacteriocin from a pseudomonad species other than *P. aeruginosa* was reported only recently (Fischer *et al.*, 2012). The activity of wheat rhizosphere isolate *P. fluorescens* SF4c, killing *P. fluorescens* CTR212, was attributed to a high-molecular mass bacteriocin whose production was upregulated by exposure of

the producer to UV light or mitomycin C. An inactive mutant was obtained by disrupting a gene predicted to be involved in tail formation (equivalent of Prf20/PA0625 in *P. aeruginosa* PAO1). By comparison with homologs present in some other *P. fluorescens* strains, this gene was presumed to reside in the intergenic region between *mutS* and *cinA*, a hotspot for the integration of prophages (Mavrodi *et al.*, 2009; Loper *et al.*, 2012). Sequence analysis of the *mutS*- and *cinA*-flanking regions revealed the presence of a *prtR* and lysis gene homolog, respectively, pointing to a potential R-type pyocin gene cluster. This genomic organization appears to be conserved in the genomic sequences of a number of other isolates, mostly originating from plant or soil environments: *P. fluorescens* strains A506, Q2-87, SS101, SBW25 (Loper *et al.*, 2012), BBc6R8 (Deveau *et al.*, 2014), F113 (Redondo-Nieto *et al.*, 2013) and Pf29Arp (Marchi *et al.*, 2013), *P. poae* RE*1-1-14 (Müller *et al.*, 2013), *P. putida* W619 (Wu *et al.*, 2011b), and *Pseudomonas* spp. Ag1 (Alvarez *et al.*, 2012), GM60, GM67 (Brown *et al.*, 2012), TKP (Ohtsubo *et al.*, 2014) and CF150 (McTee *et al.*, 2013).

The overall synteny with the *P. aeruginosa* pyocin R clusters suggests that these genes compose the genetic backbone for production of R-type tailocins. However, a number of striking differences are apparent. Of the *P. aeruginosa* regulatory genes, only a *prtR* homolog is present and it is positioned in opposite orientation, convergent with the other pyocin genes. Although well conserved among these pseudomonads themselves, these PrtR proteins share < 40% amino acid identity with *P. aeruginosa*. Together with the absence of PrtN and PrtB, this divergence suggests that the regulatory mechanisms triggering expression deviate from *P. aeruginosa* (Wu & Jin, 2005).

A significant difference is also apparent in the respective lysis cassettes. The first two genes, encoding a holin (Prf9/PA0614) and a chitinase-like lytic enzyme (Prf24/PA0629; Pfam PF00182), are conserved but the additional genes are of different origin. The *P. aeruginosa* proteins Prf25 (PA0630) and Prf26 (PA0631) are homologous to the lambdoid *P. aeruginosa* phage D3 proteins Orf32 and Orf33 (Kropinski, 2000; Canchaya *et al.*, 2003). Their functions seem to be fulfilled by two small proteins encoded by a gene pair located in different reading frames within the same nucleotide stretch (Fig. 6). These proteins are homologous to the spanins required for lysis by phage λ , the integral cytoplasmic protein Rz (Pfam PF03245) and the outer-membrane lipoprotein Rz1 (Pfam PF06085; Summer *et al.*, 2007; Berry *et al.*, 2012). Due to this unusual gene organization, only few of the Rz1-encoding genes within these R-pyocin clusters are annotated (likewise for strain SF4c). Remarkably, these spanin genes are absent from the lysis cassette of *P. fluorescens* A506 and

Pseudomonas sp. CF150. Like the overall clusters, also the Rz and Rz1 sequences have diverged considerably among the different strains. For instance, amino acid sequence identity among the seven *P. fluorescens* strains ranges from 53% to 73% for Rz and from 61% to 83% for Rz1.

Another sequence stretch lacking synteny with *P. aeruginosa* corresponds to the region between the PAO1 genes encoding Prf18 (PA0623; tail tube protein) and Prf20 (PA0625; tail length determinant). Instead, significant homology (35% amino acid identity) is found with a protein encoded by a similarly positioned gene (*orf30*) of temperate *Vibrio* phage VP882 (Lan *et al.*, 2009). Also, the tail fiber region of this plasmid-like prophage (in particular tail fiber protein Orf21 with *c.* 60% amino acid identity) shares homology with three tail protein genes in a subset of these strains (*P. fluorescens* strains A506, BBc6R8, FH4, SS101, and *P. poae* RE*1-1-14). However, all the other strains carry the corresponding phage P2-related genes, like all *P. aeruginosa* strains (Fig. 6).

The alignment also indicates that additional tailocin-associated cargo genes have been acquired by some of these strains. The *P. fluorescens* SS101 region upstream of the *cinA*-proximal lysis genes is loaded with a homolog of the *P. aeruginosa* gene encoding AmpDh3, a peptidoglycan peptidase involved in cell wall remodeling (Lee *et al.*, 2013). Genes located between *mutS* and *prtR* encode a putative toxin of the Zeta family (inhibiting peptidoglycan synthesis; Mutschler & Meinhart, 2011) and of the YafO family (inhibiting protein synthesis; Zhang *et al.*, 2009) in *P. fluorescens* strains BBc6R8 and SS101, respectively. The *mutS*–*prtR* intergenic sequences are the most variable parts of these clusters and expanded considerably in *P. fluorescens* SS101 (by 5.1 kb) and *P. fluorescens* A506 (by 6.7 kb). In the latter strain, this stretch codes for putative metabolic enzymes (a carboxylase, 2-hydroxyacid dehydrogenase, and methyltransferase).

Type VI secretion systems

One more complex competition-mediating machinery is the type VI secretion system (T6SS), of which multiple variants with differentiated functions may coexist in a particular strain. Originally identified in *Vibrio cholerae* (Pukatzki *et al.*, 2006), this system was later found to be widespread among Gram-negative bacteria, including pseudomonads. Remarkably, the T6SS not only aims at bacteria but also at eukaryotic cells. Targeted microorganisms may be from the same or a different genus. Growth inhibition by the T6SS requires cell-to-cell contact and is mediated by a syringe-like apparatus that allows the transfer of toxic effector molecules. Contrary to the contact-dependent inhibition (CDI) system (see Contact-dependent inhibition), these toxins are not covalently

coupled to their ‘carriers’. Similar to S-type pyocins and CDI effectors, the inhibitory molecules of the T6SS require immunity proteins to prevent self-inhibition (Bönemann *et al.*, 2010; Silverman *et al.*, 2012; Ho *et al.*, 2014; Russell *et al.*, 2014).

Type VI secretion (T6S) has been linked to a variety of processes, including conjugation, biofilm formation, virulence, quorum-sensing regulation and persistence in chronic infections, but these topics are not within the scope of this review.

Structural features

From a structural point of view, T6SSs share many similarities with phage-type infection systems. The T6SS consists of an inverted phage-derived syringe-like apparatus embedded in a cell envelope-spanning membrane-associated assembly. The core of the T6S machinery consists of a minimal set of 13 proteins that are required to obtain a functional architecture, most of them predicted to have a cytoplasmic localization (Filloux *et al.*, 2008; Pukatzki *et al.*, 2009; Bönemann *et al.*, 2010; Cascales & Cambillau, 2012; Silverman *et al.*, 2012; Coulthurst, 2013; Ho *et al.*, 2014; Russell *et al.*, 2014).

To date, the global T6SS ultrastructure has not been determined, although several individual T6SS components have been characterized. T6SS landmarks include an AAA+ Clp-like ATPase (ClpB/ClpV), an IcmF (intracellular multiplication protein F) homolog, and a regulatory FHA (forkhead-associated) domain protein. The phage tail-like structure consists of Hcp (hemolysin-coregulated protein), VgrG (valine/glycine repeat protein G), TssB (type six secretion B) and TssC (Cascales & Cambillau, 2012; Silverman *et al.*, 2012; Ho *et al.*, 2014). The first two proteins are secreted by the T6S machinery and share structural homology with tail tube protein gp19 and tail spike proteins gp5/gp27 from bacteriophage T4, respectively (Kanamaru, 2009; Leiman *et al.*, 2009). Hcp1 adopts a hexameric-ring structure and is the major constituent of the tube (Mougous *et al.*, 2006), while VgrG appears as a trimer at the needle tip, allowing membrane breaching and puncturing of the bacterial envelope (Pukatzki *et al.*, 2007; Hachani *et al.*, 2011). Proteins from the PAAR repeat superfamily are present at the VgrG spike and form a sharp conical extension and are involved in attachment of effector domains for translocation into target cells (Shneider *et al.*, 2013). Notably, an amino-terminal PAAR domain is present in the predicted DNase pyocin S10 (Table 1). Hcp and VgrG require a functional T6S apparatus for their release into the extracellular medium, to form the nanosyringe. TssB and TssC (HsiB/HsiC) form a Hcp tube-surrounding dynamic sheath that is able to undergo repeated cycles of extension and

contraction, meanwhile remaining attached to the inner membrane (Basler *et al.*, 2012; Lossi *et al.*, 2013; Zoued *et al.*, 2013). Energy necessary to drive the secretion of Hcp is thought to be provided by the aforementioned ClpV that disassembles the contracted T6SS sheath structure (Basler & Mekalanos, 2012). Several other components participating in the process have been identified but their exact contribution in the syringe dynamics often remains unclear. TssE (or HsiF) is a gp25-like component (Lossi *et al.*, 2011), while TagJ interacts with TssB, possibly by modulating its incorporation into the T6SS (Lossi *et al.*, 2012).

The inhibitory effect caused by the T6SS originates from its ability to deliver deleterious effector molecules into the cytoplasm or periplasm of target cells (Hood *et al.*, 2010; Russell *et al.*, 2013). Recently, it was revealed that Hcp bears a key role in this process, as the central pore of the Hcp ring is able to temporarily bind the effectors. Consequently, Hcp not only acts as a structural tail tube protein, but also functions as a chaperone exporting the T6SS toxins (Silverman *et al.*, 2013). Furthermore, the observation that several effectors may not be Hcp stabilized, but be VgrG associated instead hints to a second pathway for T6S effector export (Whitney *et al.*, 2014).

Multiplicity of T6SSs in pseudomonads

The genome of *P. aeruginosa* PAO1 encodes three T6SS loci, termed Hcp secretion island I (HSI-I), HSI-II and HSI-III, each set up by 15–20 genes. Of these, HSI-I has been studied in detail and currently serves as a model system for the study of the syringe dynamics, effector molecules and the physiological role of T6SSs. H1-T6SS, located on HSI-I, secretes at least six toxic effector molecules, Tse1 to Tse6 (Type VI secretion exported 1-2-3-4-5-6) into bacterial target cells (see next section). The other T6SSs, that is, H2-T6SS and H3-T6SS, export nonoverlapping sets of effector molecules (Bleves *et al.*, 2010; Hood *et al.*, 2010; Russell *et al.*, 2014). Next to *P. aeruginosa*, bioinformatic analysis revealed the presence of putative T6SS gene clusters in numerous other *Pseudomonas* species. Functional characterization of these systems is lacking in most cases, however. Genomic similarities and phylogenetic divergence of T6SS loci suggest an independent evolution (Sarris & Scoulica, 2011). Consequently, it is often difficult to dissect the exact roles of these T6SSs and to estimate whether they would be involved in interbacterial inhibition processes at all.

In silico analysis of the genomes of six *P. syringae* pathovars indicated the presence of one to two T6SS gene clusters, with *P. syringae* pv. *syringae* DC3000 carrying two clusters. Putative effector orthologs were identified as well, though their number was strain dependent (Sarris

et al., 2010). These results support the previous observation that several *P. syringae* strains carry T6SS core genes (Shrivastava & Mande, 2008). Two distinct T6SS genomic islands were found in genome sequence drafts of several *Pseudomonas cannabina* pv. *alisalensis* strains, one orthologous to the T6SS-II cluster of strain DC3000, while the other is more closely related to T6SS-I from *P. aeruginosa* PAO1. The absence of orthologs of Tse effectors in these strains hints to significant differences in the biological function of these systems (Sarris *et al.*, 2013). Two T6SS islands were equally found in *Pseudomonas mendocina*, homologous to HSI-I and HSI-II from PAO1, while *Pseudomonas entomophila* carries one HSI-II-like locus (Sarris & Scoulica, 2011). Genome mining of several *P. fluorescens* genomes indicated that these strains carry one to three T6SS clusters, encompassing four types in total, three of these being similar to the *P. aeruginosa* loci (Loper *et al.*, 2012). Model strain *P. protegens* Pf-5 equally carries a cluster similar to HSI-I (Hassan *et al.*, 2010; Loper *et al.*, 2012). In a broader genomic survey, comparison of 34 pseudomonad genomes indicated that virtually all *Pseudomonas* strains carry at least one T6SS locus. Clusters can be classified in five main phylogenetic groups based on comparison of 11 core genes. Specific sets of additional genes, associated with *vgrG* genes, may play a role in secretion or could be new T6S effectors (Barret *et al.*, 2011).

Functionality of a *P. syringae* T6SS has been demonstrated for strain DC3000, where Hcp2 is involved in competition of its producer with enterobacteria and yeasts. On the contrary, Hcp2 does not contribute to virulence or colonization of tomato or *Arabidopsis* plants (Haapalainen *et al.*, 2012). Biocontrol strain *P. fluorescens* Pf29Arp, that is closely related to the *P. brassicacearum*-like subgroup, carries four T6SS clusters. In the presence of necrotized wheat roots, infected with the pathogenic fungus *Gaeumannomyces graminis* var. *tritici*, expression of two *clpV* genes is significantly upregulated, but not for the other two *clpV* genes, although these are expressed as well. It is unclear however whether the upregulation of two T6SS clusters is linked with the biocontrol function of Pf29Arp (Marchi *et al.*, 2013). *Pseudomonas fluorescens* MFE01 carries at least two *hcp* genes. Mutation of *hcp2* results in reduced or abolished antagonism against a number of pseudomonads (Decoin *et al.*, 2014).

Toxic effectors and immunity proteins of T6SSs

T6S-targeted bacterial cells are killed by inhibitory effectors, transferred via the tail-like apparatus and subsequently interfering with essential processes. As mentioned before, these proteins are guided to the T6S apparatus upon binding to the Hcp chaperone. To prevent self-inhibition of the

producer, immunity proteins are available that may form complexes with their cognate partners. Genes encoding such inhibitor proteins are typically located immediately downstream of the effector protein genes and are required for producing dedicated E–I (effector/immunity) pairs (Russell *et al.*, 2014).

Secretome analysis of the H1–T6SS of *P. aeruginosa* PAO1 revealed three substrates that are secreted under tight posttranslational control, Tse1–2–3 (Hood *et al.*, 2010). Recently, it was found that three more effectors are associated with H1–T6SS, hence called Tse4, Tse5, and Tse6 (Whitney *et al.*, 2014). Tse1 and Tse3 (Fig. 9) are targeted to the periplasm, both acting as peptidoglycan-hydrolyzing enzymes (Russell *et al.*, 2011). Their mechanism of action is quite different, however. Tse1 belongs to the superfamily of NlpC/P60 endopeptidases and cleaves the γ -D-glutamyl-L-meso-diaminopimelic acid of peptidoglycan in target cells (Russell *et al.*, 2011; Benz *et al.*, 2012; Chou *et al.*, 2012; Ding *et al.*, 2012; Zhang *et al.*, 2012b). In addition to Tse1, three other amidase effector groups, able to cleave within the peptide stem of peptidoglycan and its cross-links, have been identified via a heuristic approach. The preferred cleavage sites of these Type VI amidase effector (Tae) proteins vary, however, suggesting that effector production may depend on the peptidoglycan structure of the targeted organisms (Russell *et al.*, 2012). Next to Tae1 (or Tse1), two additional Tae structures have been solved. Tae3 in complex with Tai3 (Type VI amidase immunity, Fig. 9) from *Ralstonia pickettii* 12D occurs as a heterohexamer, composed of two Tae3 proteins and two Tai3 heterodimers (Dong *et al.*, 2013), while Tae4/Tai4 from *Salmonella* Typhimurium LT2 and *Enterobacter cloacae* ATCC13047 (Fig. 9) occur as a heterotetramer of two Tae4 molecules and a Tai4 dimer (Benz *et al.*, 2013; Zhang *et al.*, 2013). Tse3 that anchors to the target membrane in a calcium-dependent

way (Lu *et al.*, 2014), adopts a goose-type lysozyme-like structure and acts as a muramidase that cleaves the β -1,4 bond between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) in peptidoglycan (Li *et al.*, 2013b; Lu *et al.*, 2013; Wang *et al.*, 2013). In *P. protegens* Pf-5, a peptidoglycan glycoside hydrolase effector not orthologous to Tse3 was identified via an informatics search and characterized. This effector protein is involved in inhibition of *P. putida* KT2440 (Whitney *et al.*, 2013). Immunity to the Tse1 and Tse3 effectors is provided by Tsi1 (Type VI secretion immunity 1) and Tsi3, respectively, present in the periplasm of the producer (Russell *et al.*, 2011; Benz *et al.*, 2012; Ding *et al.*, 2012; Shang *et al.*, 2012). Tse2, protected by a Tsi2 dimer, causes quiescence but not death, in recipient cells by an unknown mechanism, possibly by exerting nuclease activity. As Tsi2 acts as a cytoplasmic neutralizer, Tse2 is thought to exert its function in the cytoplasm of the target cells (Hood *et al.*, 2010; Li *et al.*, 2012; Zou *et al.*, 2012). Tsi1–3 are not exported and reside in the periplasm of the producer, while Tsi2 is complexed with Tse2 and dissociates prior to or during the secretion process (Hood *et al.*, 2010; Russell *et al.*, 2011; Li *et al.*, 2012). Less is known about effectors Tse4, Tse5, and Tse6, protected by Tsi4–5–6, respectively, mainly due to a lack of homology with previously characterized proteins (Whitney *et al.*, 2014). Tse4 is Hcp stabilized, similar to Tse1, Tse2, and Tse3, and acts in the periplasm via an unknown mechanism. Homologs of this effector/immunity pair are also encoded in the genomes of several other pseudomonads, such as *P. brassicacearum* NFM421, *P. fluorescens* F113, and *P. protegens* Pf-5, but their functionality remains to be demonstrated. Tse5 and Tse6 are PAAR repeat-containing proteins interacting with a cognate VgrG, but not with Hcp, for host export. Tse5 exerts its toxic function in the periplasm, in contrast to Tse6 that acts as a potent

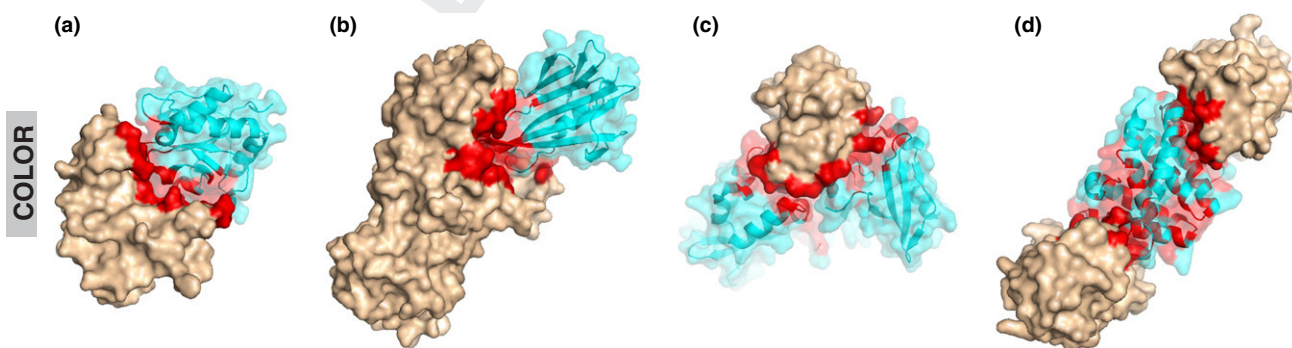


Fig. 9. Structures of T6SS effectors/immunity protein complexes. Effector proteins are colored wheat (surface), immunity proteins cyan (ribbons with transparent surface). Residues involved in toxin/immunity protein interactions are colored red. (a) Tse1–Tsi1 from strain PAO1 (PDB 4FQB), (b) Tse3–Tsi3 from strain PAO1 (PDB 4M5F), (c) Tae3 in complex with a Tai3 heterodimer as part of a heterohexamer (not shown) from *Ralstonia pickettii* 12D (PDB 4HZ9), (d) heterotetramer of two Tae4 proteins and a Tai4 dimer from *Enterobacter cloacae* ATCC13047 (PDB 4HFK).

cytoplasmic toxin. Inhibitory domains of Tse5 and Tse6 are located at their carboxy-terminal termini, in the latter case carrying a putative Toxin 61 domain. The presence of Rhs/YD-repeats in Tse5 suggests that its secretion proceeds via the T6S pathway (see Rhs elements as mediators of intercellular competition).

As substrates of *P. aeruginosa* PAO1 H2-T6SS, a superfamily of phospholipase effectors was identified. These enzymes probably target phospholipids that are accessible from the periplasm, as associated genes encoding for immunity partners are predicted periplasmic proteins. Antibacterial activity of the lipase effector PldA (or Tle5, Type VI lipase effector) is effectuated by the degradation of phosphatidylethanolamine, the major phospholipid in bacterial membranes. Immunity to Tle5 is granted by Tli5 (Russell *et al.*, 2013). In addition, the H2-T6SS is also required for bacterial internalization into epithelial cells and plays a role in worm virulence (Sana *et al.*, 2012). In contrast to H1-T6SS and H2-T6SS, no role in interbacterial competition could yet be attributed to H3-T6SS, a system that also contributes to virulence in a worm model (Sana *et al.*, 2013). In *P. aeruginosa* PA14, H2-T6SS and H3-T6SS can compensate for one another in mouse virulence (Lesic *et al.*, 2009).

Regulation of T6SSs in *Pseudomonas*

Transcriptional regulation

In *P. aeruginosa* PAO1, the three T6SS loci are regulated in a different way. The latter is in line with their distinct evolutionary origins and the different roles these systems may fulfill. Cluster-dependent regulation also suggests that these systems act in completely different contexts (Bingle *et al.*, 2008). Transcription of T6SSs depends on a myriad of factors, including environmental, QS and other bacteria-derived signals (Silverman *et al.*, 2012). These topics will be briefly summarized, but the reader is referred to more specialized reviews for HSI regulation (Miyata *et al.*, 2013).

QS was found to play a major role in HSI gene cluster regulation. It represses HSI-I, but activates the co-regulated HSI-II and HSI-III (Lesic *et al.*, 2009; Sana *et al.*, 2012; Sana *et al.*, 2013). The RpoN σ^{54} factor causes different effects on the T6SS operons. RpoN activates the expression of one of the H3-T6SS operons (left), but represses the other (right) and H2-T6SS (Sana *et al.*, 2013), this negative regulation being rather atypical for T6SSs (Bernard *et al.*, 2011). In addition, the Sfa3 enhancer-binding protein of H3-T6SS has no effect on RpoN-mediated transcription, while the repression of H2-T6SS by RpoN is Sfa2-dependent (Sana *et al.*, 2013). Next to this, HSI-I is regulated by two-

component systems and small RNAs (sRNAs) (Goodman *et al.*, 2004; Mougous *et al.*, 2006). Mutation of the *subB* gene causes an upregulation of GacA and the downstream sRNAs, RsmY, and RsmZ, which triggers T6SS in *P. aeruginosa* PAK (Li *et al.*, 2013a). Similarly, GacA was found to upregulate expression of different T6SSs in the rhizobacterium *P. aeruginosa* M18 (Wei *et al.*, 2013). Mutation of the sensor RetS causes Hcp1 expression in PAK via enhanced c-di-GMP signaling (Moscoso *et al.*, 2011). RetS forms a heterodimer with GacS, hereby preventing the autophosphorylation of the latter. Consequently, phosphotransfer-dependent activation of GacA cannot occur (Brencic *et al.*, 2009). Next to RetS, sRNA production via GacA is also controlled by HptB via a complex regulatory cascade. Interestingly, HptB only regulates *rsmY* expression (Bordi *et al.*, 2010). Furthermore, HSI-II is also regulated by iron. In its promoter region, two putative Fur boxes are present, causing negative regulation by iron (Sana *et al.*, 2012). Interestingly, the H1-T6SS is assembled in response to foreign T6SS-mediated attack from hostile bacteria (Basler *et al.*, 2013). This counterattack measure is also adopted upon gene transfer from Type IV secretion systems (Ho *et al.*, 2013).

A number of other conditions have been associated with altered T6SS levels, though they are often the result of indirect effects. Subinhibitory concentrations of kanamycin induce the expression of H1-T6SS in *P. aeruginosa* PAK. This is only observed when a functional Gac/Rsm pathway is present (Jones *et al.*, 2013). In the host-adapted, alginate-overproducing *P. aeruginosa* 2192, the presence of human respiratory mucus triggers expression of the HSI-I gene cluster (Cattoir *et al.*, 2013). In *P. syringae* B728a, but not in strain DC3000, expression of T6SS can be induced by an osmotic upshift (Freeman *et al.*, 2013). In *P. fluorescens* Pf0-1, a soil-induced gene product predicted to be a component of a T6SS (Pfl01_5595) was shown important in arid soil persistence, underlining the role of microbial interactions in natural environments (Varivarn *et al.*, 2013).

Posttranslational regulation

At posttranslational level, H1-T6SS functioning is strictly modulated by the phosphorylation state of Fha1. This is controlled by the transmembrane serine-threonine Hanks-type kinase PpkA. When Fha1 is phosphorylated, Hcp1 secretion will be triggered. The PP2C-type phosphatase PppA antagonizes the phosphorylation of Fha1 (Mougous *et al.*, 2006). PpkA contains autophosphorylation sites and its kinase activity is positively regulated by TagR (Hsu *et al.*, 2009). In addition to this, three other PpkA/PppA checkpoint regulators, TagT–TagS–TagQ, were

found, with TagTS forming an ATP-binding cassette (ABC) transporter complex. Study of their cellular localization suggests that these four proteins participate in a trans-membrane signaling pathway (Casabona *et al.*, 2013). TagF functions as a posttranslational repressor, acting independently from the threonine phosphorylation pathway (Silverman *et al.*, 2011).

Contact-dependent inhibition

CDI represents another strategy of bacteria to protect themselves from rival bacteria. CDI⁺ bacteria own a variety of toxins that allow growth inhibition of neighboring competitors via direct cell-to-cell contact. Similar to S-type pyocins and effectors of T6SSs, producers need to temporarily inactivate inhibitory domains by forming CDI toxin/immunity protein pairs. CDI systems belong to the type V two-partner secretion systems (TPS; Aoki *et al.*, 2011; Ruhe *et al.*, 2013a; Hayes *et al.*, 2014). CDI has been well studied in *E. coli* and *Burkholderia*, but the exact nature of this inhibitory system in *Pseudomonas* remains poorly understood.

Similar to T6SS-mediated antagonism, CDI is thought to play a role beyond bacterial competition. In the model strain *Burkholderia thailandensis* E264, CDI proteins were associated with autoaggregation and adherence to an abiotic surface (Anderson *et al.*, 2012) and with biofilm formation (Garcia *et al.*, 2013). Bacteria may use CDI in cooperative behaviors to build communities and to prevent non-self bacteria from entering a biofilm community (Garcia *et al.*, 2013).

Genetic organization of CDI systems

CDI mediated by the CdiB/CdiA system was initially observed in *E. coli* isolate EC93. The antibacterial effect is caused by expression of the *cdiBAI* gene cluster, which is sufficient to confer the CDI⁺ inhibitor phenotype to *E. coli* K-12 cells (Aoki *et al.*, 2005). CdiB and CdiA are members of the TpsB and TpsA families of TPS proteins, respectively. TpsB is an outer-membrane β -barrel protein that facilitates the export of TpsA across the outer membrane. The latter is a very large hemagglutinin-repeat protein that extends far from the cell surface (Mazar & Cotter, 2007). The growth-inhibitory effect of CdiA is caused by a toxin domain, located at its C-terminus, called CdiA-CT. Overall, CdiA homologs share large regions of sequence homology, but their carboxy-terminal regions (c. 300 AA) diverge abruptly after a common VENN peptide motif (PF04829), suggesting that many different toxins (CdiA-CTs) in CDI⁺ strains exist. These findings also imply that CDI⁺ bacteria exploit a common secretion mechanism (Aoki *et al.*, 2010).

In *Burkholderia*, the CDI operon organization is different, constituted as *bcpAIOB* (*Burkholderia* CDI proteins A, I, O and B). The additional small protein BcpO was predicted to be a lipoprotein (Anderson *et al.*, 2012). Sequence divergence of the *Burkholderia* cytotoxic domains is observed after a different conserved Nx(Q/E) LYN motif (Anderson *et al.*, 2012; Nikolakakis *et al.*, 2012).

Receptor binding of CdiA proteins

CdiA initially interacts with the BamA receptor of CDI-sensitive *E. coli* cells (Aoki *et al.*, 2008). BamA is an essential, highly conserved protein participating in the β -barrel assembly machinery (BAM complex), required for the appropriate integration of proteins into the outer membrane (Webb *et al.*, 2012). Replacement of *bamA* alleles with the *E. coli* MC4100 *bamA*, sensitizes other enterobacteria to the *E. coli* EC93 system CDI^{EC93}. *Vice versa*, several heterologous *bamA* genes confer CDI^{EC93} resistance on *E. coli*. Sequence variation among enterobacterial BamA orthologs seems concentrated to three surface-exposed loops. Scrutiny of these stretches showed that the CdiA^{EC93}-binding epitope is defined by the combination of two of these loops (loops 6 and 7). Though BamA sequence variation is quite high, only a limited number of loop sets seem to exist, suggesting that BamA^{*E. coli*} and CdiA^{EC93} fulfill a key role in self-nonself discrimination (Ruhe *et al.*, 2013b). Using CdiA-specific antibodies it was found that CdiA from *E. coli* 536 is deposited onto the surface of target bacteria. The CdiA-CT is translocated into target cells, while the amino-terminal region stays at the surface, suggesting cleavage prior to translocation (Webb *et al.*, 2013). One cleavage site is located at the amino-terminus, likely removing the signal peptide, while (at least) two other cleavage sites are situated within the carboxy-terminal portion of CdiA (Aoki *et al.*, 2005). Furthermore, AcrB was identified as a potential CdiA downstream target (Aoki *et al.*, 2008). Located in the inner membrane, this protein is part of a distinct multicomponent machine, exporting small molecules into the extracellular environment, hereby acting as a multidrug resistance pump (Nikaido & Pagès, 2012). At this point it is still unclear however how the CdiA effector would exploit AcrB to deliver its toxin domain (Aoki *et al.*, 2008).

Interestingly, knocking out additional proteins of the Bam and Acr multi-component machines does not affect CDI. This suggests that inhibition resulting from interactions between (parts of) CdiA with BamA or AcrB originate from novel, import-related functions of the latter proteins (Aoki *et al.*, 2008). Key interaction partners for binding of CdiAs in other genera than *E. coli* remain undisclosed at this moment.

Biological activity of CdiA-CTs

The toxicity function of a CdiA is located at its C-terminus, therefore termed CdiA-CT. This small domain is bound and inactivated by a cognate immunity protein, called CdiI, protecting the CDI⁺ cells from auto-inhibition. Sequence heterogeneity suggests a wide range of toxic activities being displayed by CdiA-CTs (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012). The carboxy-terminal domain of CdiA₃₉₃₇₋₂ from *Dickeya dadantii* 3937 shares 35% identity with the pyocin S3 nuclease domain and was confirmed to exert DNase activity. On the contrary, the toxic domain of *E. coli* 536 cleaves tRNA (Aoki *et al.*, 2010). In *Burkholderia pseudomallei* ten different CDI sequence types were identified based on polymorphisms within the *cdiA-CT/cdiI* coding regions. Three of these were found to display tRNase activity, based on their similarities with the cytotoxic domain of colicin E5 (Nikolakakis *et al.*, 2012). HecA (or CdiA^{EC16}) from *D. dadantii* EC16 carries a carboxy-terminal domain that resembles the rRNase from colicin E3 (Walker *et al.*, 2004). Crystal structures of the CdiA-CT/CdiI complexes of a Zn²⁺-dependent DNase from *E. coli* EC869 and the tRNase from *B. pseudomallei* 1026b were solved, showing that each immunity proteins binds and inactivates its cognate toxin in a unique way (Morse *et al.*, 2012). Bioinformatic analysis predicts that several CDI toxins are nucleases, adenosine deaminases, ADP-ribosyl cyclases or metallopeptidases. Despite low sequence homology between CdiA-CTs, their structures may be quite similar in the end (Zhang *et al.*, 2011).

Interestingly, certain CdiA-CT/CdiI pairs are found in diverse bacterial clades. This suggests that *cdiA-CT/cdiI* gene pairs can be horizontally exchanged between bacteria. The modular nature of CdiAs is underlined by the observation that chimeric CdiAs are functional (Aoki *et al.*, 2010). Additional *cdiA-CT/cdiI* gene pairs are often located downstream of the main *cdi* cluster. These toxin/immunity proteins pairs are called orphan modules as they seem displaced from their full-length CdiAs. As such these orphans do not encode functional CdiAs, though they may provide a reservoir for CdiA-CT/CdiI variation. They often contain insertion sequence elements and transposon-related genes, pointing to horizontal gene transfer as well (Poole *et al.*, 2011).

CdiI as a protector from auto-inhibition

The *cdiI* gene is tightly linked to *cdiA* and encodes a small immunity protein that protects CDI⁺ cells from auto-inhibition (Aoki *et al.*, 2005). Structures of two different CdiA-CT/CdiI complexes indicate that the interaction between both partners causes an occlusion of the

active site, though the way this is achieved may be somehow different (Morse *et al.*, 2012). The presence of a functional CdiI is sufficient to protect the strain from autoinhibition (Aoki *et al.*, 2009). CdiI immunity proteins are specific for their cognate CdiA-CTs but may display a high level of variability. Therefore, proper immunity will only be provided if a suitable antitoxin partner is present. Consequently CDI concerns a network of toxin/immunity protein pairs, each having the potential to mediate inter-strain competition (Aoki *et al.*, 2010; Nikolakakis *et al.*, 2012).

In *D. dadantii* EC16, a CDI immunity protein called VirA has been associated with plant virulence (Rojas *et al.*, 2004). This effect could be explained in *D. dadantii* 3937, where a *virA* mutant is outcompeted when grown together with the wild-type strain on chicory (Aoki *et al.*, 2010). Such mutant is viable under laboratory conditions but on plants expression of the associated toxin gene is induced, resulting in cell death due to the lack of protection by the cognate immunity protein.

Putative CDI systems of pseudomonads

The presence of diagnostic structural motifs (hemagglutinin repeats; Pfam families PF05594 and PF13332), secretory signature motifs (hemagglutination activity domain, PF05860) and carboxy-terminal border region (VENN motif, PF04829), along with well-conserved tripartite organization assists in recognizing potential CDI gene clusters in pseudomonads (Table 2, Table S2). These genomic regions are often poorly annotated due to the polymorphic nature of the CdiA and CdiI proteins and the low level – or even lack – of discernable sequence homology for the relatively short CdiA-CT domains and the small-sized the CdiI proteins (Poole *et al.*, 2011).

In addition to *D. dadantii*, some *P. syringae* strains, as well as a few other phytopathogenic bacteria, are equipped with a gene cluster for a putative CDI system with DNase activity related to pyocin S3: *P. syringae* pv. *theae* ICMP 3923 (partially sequenced CdiA of > 1383 amino acids), *Xanthomonas axonopodis* pv. *citri* 306 (CdiA of 4753 amino acids; da Silva *et al.*, 2002), and *Pantoea ananatis* LMG 5342 (CdiA of 3728 amino acids; De Maayer *et al.*, 2012). Sequence comparison of the corresponding predicted activity domains of CdiA and of the CdiI proteins (all in the range of c. 140–145 amino acids) supports an evolutionary relationship with the DNase and immunity modules of S3-type pyocins (Figs S2 and S3). A close homolog of the ICMP 3923 CdiA protein is encoded by the *P. syringae* pv. *syringae* SM genome (full-length protein of 6173 amino acids; GenBank Accession Number EPF67127), but the pyocin S3-like carboxy-terminal sequence is replaced by a domain of unknown

Table 2. Predicted CDI toxins with known CT domains and corresponding immunity proteins in *Pseudomonas* genomes

CDI protein			Immunity protein*			
Name†	Size‡	CT domain§	Name†	Size‡	Domain§	Strains
<i>Burkholderia</i> -like deaminase	5207	PF14424	Imm2	117	PF14426	<i>P. aeruginosa</i> 2192
<i>Burkholderia</i> -like tRNase	3213–3563	–	–	130	–	<i>P. aeruginosa</i> PA7, <i>Pseudomonas</i> sp. M1
ColD-like tRNase	5688	PF11429	Colicin immunity	90	PF9204	<i>P. fluorescens</i> FH5
Endo U nuclease	5627–5644	PF14436	–	84–122	–	<i>P. aeruginosa</i> PAO1, <i>P. aeruginosa</i> SCV20265
HNH-AHH nuclease	3074–3092	PF14412	–	135–204	–	<i>P. brassicacearum</i> subsp. <i>brassicacearum</i> NFM421, <i>P. fluorescens</i> F113, <i>P. fluorescens</i> Q8r1–96
HNHc nuclease	2776	SM000507	DUF2465	156	PF14085	<i>P. fluorescens</i> NZ052
HNH-HHH nuclease	4058	PF15637	–	143	–	<i>P. fluorescens</i> SBW25
HNH-LHH nuclease	4310	PF14411	SUKH–5	139	PF14567	<i>Pseudomonas</i> sp. VLB120
MafB19 deaminase	3484–3871	PF14437	–	129–133	–	<i>P. aeruginosa</i> BL08, <i>P. aeruginosa</i> VRFP08
Pyocin S3-like DNase	> 1383	–	–	141	–	<i>P. syringae</i> pv. <i>theae</i> ICMP 3923
Restriction endonuclease fold7	2772–4196	PF15469	–	248–250	–	<i>P. fluorescens</i> SBW25, <i>P. fluorescens</i> SS101, <i>P. fulva</i> 12-X

GenBank Accession Numbers are provided in Table S2.

*Product of gene located immediately downstream of toxin gene.

†Based on domain present in CT module; –, not assigned.

‡Number of amino acids or size range of different proteins.

§Pfam (PF) or SMART (SM); –, not defined.

function, illustrating the polymorphic nature of such toxins. In the unannotated region downstream of *P. syringae* SM *cdiA* not only the putative cognate immunity gene is present, but also an orphan *cdiA*-CT/*cdiI* module. This gene pair encodes a small CdiA-like protein (only 155 amino acids) and immunity protein nearly identical in sequence to the CdiA-CT/immunity protein pair of *P. syringae* ICMP 3923 (94% and 93%, respectively).

Another example of apparent recruitment of a CdiA-CT/immunity module from a bacteriocin family is found for a putative CDI system of *P. fluorescens* FH5 (Rhodes *et al.*, 2013; Fig. 1). Phylogenetic analysis of the CT domain assigns these CdiA/CdiI components (5688/90 amino acids) to the colicin D family, suggesting that this CDI system involves degradation of tRNA in target cells (Figs S4 and S5). The considerable sequence divergence from other colicin D members, including the novel pyocins S11 and S12, hints to probable differences in tRNA substrate specificities. In addition, a few orphan immunity proteins encoded by genes apparently lacking an upstream-located cognate tRNase toxin gene were identified in *P. syringae* pv. *tomato* strains T1 (conserved but not annotated in at least three other strains of this pathovar), in some other *P. syringae* isolates of unspecified pathotype (UB246, USA011), in the mushroom pathogen *Pseudomonas gingeri* NCPPB 3146, and in rhizosphere isolate *P. fluorescens* Q8r1-96 (Fig. S5). Two of these immunity genes are located in the polymorphic immunity regions downstream of an intact CDI system (*P. fluorescens*

Q8r1–96) or conserved Rhs system (*P. syringae* USA011; see Rhs elements as mediators of intercellular competition), both of unknown cytotoxic type, while others are still associated with sequence remnants of a CDI system (*P. syringae* pv. *tomato* T1) or putative S-type pyocin (*P. gingeri* NCPPB 3146).

The lack of functional data for CDI-like proteins of pseudomonads does not preclude that they may be of importance for interactions between *Pseudomonas* strains and other bacteria. Bioinformatic analysis of polymorphic cytotoxins has revealed the existence of a multitude of toxin modules and immunity proteins, in particular systems that affect nucleic acids (Zhang *et al.*, 2011, 2012a, b). Inspection of pseudomonad genomes for gene clusters encoding CdiA systems with a discernable type of toxin and immunity domain (based on Pfam and SMART toxin domains) reveals a considerable number of such putative strain-specific toxins, suggesting they also play a significant role in the social life of pseudomonads (Table 2). In addition to the pyocin-like modules targeting DNA or RNA already described, 11 different types of CdiA proteins were identified, all of them targeting nucleic acids. Many of these belong to recently defined DNase subfamilies of the HNH superfamily (Zhang *et al.*, 2012a). Additional CdiA proteins are encoded by pseudomonad genomes, but their carboxy-terminal part and immunity partner protein lack a match to a defined protein family domain. This is for instance the case for CdiA proteins from *P. aeruginosa* PA7 (3563 amino acids) and

Pseudomonas sp. M1 (3213 amino acids) that show low but significant homology to a novel type of tRNase domain identified in *B. pseudomallei* 1026b (Table 2; Fig. S9; Morse *et al.*, 2012; Nikolakakis *et al.*, 2012).

Rhs elements as mediators of intercellular competition

Rearrangement hotspot (*rhs*) elements were initially identified in *E. coli* as genomic sites that promote recombination, based on their role in chromosome duplications (Lin *et al.*, 1984). Later, these elements were detected in numerous other bacterial genera, including *Pseudomonas*. From a structural point of view, Rhs proteins share similarities with CdiA proteins, though the mechanism required for the secretion of the former seems to be different (Hayes *et al.*, 2014). Similar to T6SSs, Rhs proteins have been associated with both bacterial/bacterial and bacterial/host interactions.

Structural features of Rhs proteins

Rhs proteins are large and typically comprise c. 1500 residues in Gram-negative bacteria. They are composed of a central repeat region constituted from tyrosine/aspartate (YD) repeat units (PF05593), unrelated to the hemagglutinin repeats from CdiA. This large domain is encoded by a GC-rich *rhs* region and is called the core. The number of YD repeats may vary, but overall this region displays a high degree of intra- and interspecies sequence conservation (Jackson *et al.*, 2009). In front of the core, at the amino-terminus, a clade-specific domain is located. The carboxy-terminal domains of Rhs proteins are termed 'tips' and are separated from the core by a highly conserved 61-amino acid region. In Enterobacteriaceae, the latter is typified by a PXXXXDPXGL sequence at the end. The Rhs carboxy-terminal domains, referred to as Rhs-CTs, are encoded by GC-poor sequences and several of them share features with the toxin modules of CdiAs (Jackson *et al.*, 2009). Comparative genomics indicated that the Rhs core domains and variable carboxy-terminal modules are evolutionarily decoupled, hinting that *rhs* diversity originates from recombination events (Jackson *et al.*, 2009; Zhang *et al.*, 2011). Interestingly, the toxin domain-encoding 3'-ends are also followed by small genes encoding self-immunity factors, called RhsI proteins, recognizing specifically their cognate toxin partners, similar to CdiA-CdiI and pyocin-type bacteriocin/immunity pairs (Poole *et al.*, 2011; Koskiniemi *et al.*, 2013). Furthermore, *rhs* loci are frequently followed by orphan modules, similar to the *cdiA-CT/cdiI* toxin/immunity gene pairs (see Biological activity of CdiA-CTs; Poole *et al.*, 2011; Zhang *et al.*, 2011). Generation of functional chimeric genes

through recombination between such orphan *rhs-CT* and the main *rhs* can provide a competitive advantage, as was recently demonstrated in *Salmonella* Typhimurium LT2 populations subjected to experimental evolution in liquid culture and within a mouse host (Koskiniemi *et al.*, 2014). Recent work indicates that Tse5, a Rhs protein from *P. aeruginosa* PAO1, requires the T6SS because it is dependent on ClpV/VgrG4 for its secretion (see Toxic effectors and immunity proteins of T6SSs in *Pseudomonas*; Whitney *et al.*, 2014). Similar to this, RhsA and RhsB from *D. dadantii* 3937 have been associated with VgrGs for proper export (Koskiniemi *et al.*, 2013).

Inhibitory activities of Rhs systems

The inhibitory function of Rhs proteins has been demonstrated in numerous studies, both in interbacterial and in bacterial/host interactions. Expression of the carboxy-terminal fragment of *rhsA* from *E. coli* initiates a toxic cellular effect. This effect could be impeded by the coexpression of a downstream gene (Vlazny & Hill, 1995), apparently functioning as an antitoxin module. Subsequently, Aggarwal and Lee demonstrated that the RhsA function interferes with transcription and translation, though the exact mechanism of its action remains unknown (Aggarwal & Lee, 2011). In *P. savastanoi* pv. *savastanoi* ITM317, an *rhs*-like genetic element was found to confer bacteriocin-like activity against multiple strains of the same pathovar. A highly homologous YD-repeat protein is also encoded by *P. syringae* pv. *syringae* B728a, though the respective Rhs-CTs share no discernable similarities. Analysis of the molecular size of the *P. savastanoi* inhibitor indicates that this Rhs protein is proteolytically cleaved (Sisto *et al.*, 2010), reminiscent of the processing observed for CdiA proteins (see Receptor binding of CdiA proteins). Two Rhs proteins from *D. dadantii* 3937 and the distantly related cell wall-associated protein A (WapA) from *Bacillus subtilis* 168 carry polymorphic carboxy-terminal toxin domains that are accompanied by neutralizing immunity proteins (RhsI and WapI, respectively; Poole *et al.*, 2011; Koskiniemi *et al.*, 2013). These Rhs proteins are deployed to sustain growth inhibition of neighboring cells. The *D. dadantii* proteins harbor DNase domains, whereas WapA acts by tRNase activity on tRNA^{Arg}. Interestingly, the secretion route of these proteins seems to be different. WapA appears to follow the general secretory pathway, directed by the presence of a cleavable Sec-secretion signal sequence, whereas the *rhs* genes from *Dickeya* are linked to *hcp* and *vgrG* genes, pointing toward T6SS-mediated translocation (see Structural features of Rhs proteins). As the T6S apparatus typically transports smaller effectors, cleavage of the Rhs protein may be required at some point (Koskiniemi *et al.*,

2013). Similar to *Dickeya*, a *rhs* element accompanied by a *hcp* and a *vgrG* gene, was found in *P. aeruginosa* PA14. There was no evidence, however, that this Rhs protein was an effector of the associated T6SS (Jones *et al.*, 2014).

In the enterohemorrhagic strain *E. coli* O26:H⁻, it was found that intestinal colonization of calves is disturbed in an *rhsA* insertion mutant (van Diemen *et al.*, 2005). The presence of a *rhs* gene in an endosymbiotic bacterium of aphids was associated with increased survival following attack by parasitic wasps (Degan & Moran, 2008). XadM, a Rhs family protein identified in *Xanthomonas oryzae* pv. *oryzae* BXO43, with several homologs in other *Xanthomonas* and *Burkholderia* strains, is required for attachment, biofilm formation and virulence to rice. Its carboxy-terminus displays similarity to a domain of unknown function (NLPC_P60) detected in several lipoproteins (Pradhan *et al.*, 2012). In *P. aeruginosa* PSE9, the *rhsT* gene located on the genomic island PAGI-9 of *P. aeruginosa* PSE9 encodes a virulence protein that is delivered to eukaryotic cells and activates the inflammatory (Kung *et al.*, 2012). The presence of two conserved histidines in the toxic domain of RhsT suggests that this protein acts as a RNase A-like metal-independent nuclease (Zhang *et al.*, 2012a). These different studies underline the diverse role of Rhs proteins as secreted toxins but also add to the role of these systems in bacterial pathogenesis.

Rhs polymorphic domains in pseudomonads

Functional data on Rhs proteins from pseudomonads is still limited at this point. Consequently, their role in interbacterial competition processes is hard to predict. Similar to CdiA proteins, a multitude of cytotoxic domains appears to be encoded by these Rhs elements, primarily affecting nucleic acids (Zhang *et al.*, 2011, 2012a). Scrutiny of pseudomonad genomes for gene clusters encoding Rhs systems with a known type of toxin and immunity domain (Pfam and SMART databases) indicates the presence of a large array of strain-specific toxin modules (Table 3). Although it cannot be excluded that some of the Rhs proteins (also) play a role in interaction of pseudomonads with a eukaryotic host (as demonstrated for the virulence factor RhsT of *P. aeruginosa* PSE9, Kung *et al.*, 2012), most likely their primary function lies in competition with other bacteria. In addition to the pyocin-like modules targeting DNA or RNA and putative nuclease-active CDI systems already described, 20 different types of Rhs proteins were identified, nearly all of them targeting nucleic acids (Table 3, Table S2). Many of these belong to recently defined DNase families of the HNH superfamily (Zhang *et al.*, 2012a). Although the *rhs*

loci are often incompletely annotated, it is clear that much more Rhs proteins are encoded by pseudomonad genomes. However, their highly diverse carboxy-terminal domain and immunity partner protein often cannot be assigned to a particular protein family.

Pseudomonas fluorescens NCIMB 11764 carries a Rhs protein with a carboxy-terminal Toxin 61 domain, the latter homologous to the *P. aeruginosa* Tse6-CT domain (Whitney *et al.*, 2014). It appears that some of the toxin modules of Rhs proteins share a common ancestry with certain S-type pyocins. A HNH domain predicted to confer DNase activity is present in a Rhs protein of *P. syringae* pv. *syringae* SM but its immunity partner lacks discernable homology with those of the HNH-type pyocins (Table 3). The RhsB protein of another phytopathogen, *D. dadantii* 3937, is equipped with a similar carboxy-terminal module/immunity protein combination and mediates T6SS-dependent intercellular competition relying on its capacity to degrade target cell DNA (Koskinen *et al.*, 2013). A Rhs protein bearing a pyocin S6/S7-related rRNase domain and cognate immunity protein was identified in *P. putida* S16 (Yu *et al.*, 2011). By one of the *rhs* loci of *P. entomophila* L48 (Vodovar *et al.*, 2006), a colicin D-like Rhs protein/immunity pair (putative tRNase activity) is encoded (Table 3; Fig. 1). The L48 Rhs cytotoxic domain is most similar to the CdiA-CT domain of *P. fluorescens* FH5, but quite distant from the tRNase modules of pyocins S11 and S12, suggesting a different ancestry for the enzymatic modules present in these pyocins, on the one hand, and those incorporated in the polymorphic toxins, on the other hand (Fig. S5). The colicin D-type of immunity gene was also detected in *P. syringae* USA011, located in the polymorphic region downstream of the genes encoding a conserved Rhs system of unknown cytotoxic type. Apparently, acquiring and maintaining individual immunity genes may contribute to protection against toxins from competitors.

Lectin-like bacteriocins

The first lectin-like bacteriocin was identified in banana rhizosphere isolate *P. putida* BW11M1 as a secreted heat- and protease-sensitive chromosome-encoded antibacterial protein, able to kill other plant-associated *Pseudomonas* strains (Parret *et al.*, 2003). Similar bacteriocins were characterized in *P. protegens* (Parret *et al.*, 2005), *P. syringae* (Ghequire *et al.*, 2012b) and *P. aeruginosa* (McCaughy *et al.*, 2014), though sequence homology proved to be low. Contrary to S-type pyocins, lectin-like bacteriocins do not bear a known cytotoxic domain, nor do they require an immunity protein to prevent autoinhibition (Parret *et al.*, 2003).

Table 3. Predicted Rhs toxins with known CT domains and corresponding immunity proteins in *Pseudomonas* genomes

Rhs protein			RhsI*			Strains
Name†	Size‡	CT domain§	Name†	Size‡	Domain§	
ColD-like tRNase	1502	PF11429	Colicin immunity	96	PF9204	<i>P. entomophila</i> L48
ColE3-like 16S rRNase	417	PF09000	–	84	–	<i>P. putida</i> S16
DYW deaminase	410	–	Imm5	193	PF14423	<i>P. syringae</i> pv. <i>glycinea</i> B076, <i>P. syringae</i> pv. <i>phaseolicola</i> 1448a
HNH-AHH nuclease	1107–1400	PF14412	–	176–199	–	<i>P. plecoglossicida</i> NB2011, <i>P. putida</i> DOT-T1E, <i>P. putida</i> F1, <i>P. putida</i> ND6, <i>P. syringae</i> BRIP39023
HNHc nuclease	881	SM000507	–	158	–	<i>P. syringae</i> pv. <i>syringae</i> SM
HNH-EHHH nuclease	1569–1603	PF15657	Imm30	130–134	PF15549	<i>P. fluorescens</i> SBW25, <i>P. fluorescens</i> SS101, <i>P. poae</i> RE*1-1-14, <i>P. synxantha</i> BGR33, <i>P. syringae</i> ICMP 18806
HNH-GH-E nuclease	1399	PF14410	YjcQ-like	108	PF09639	<i>P. syringae</i> BRIP34881
HNH-GHH nuclease	1484	PF15636	SUKH-1	199	PF09346	<i>Pseudomonas</i> sp. TKP
HNH-GHH2 nuclease	1404	PF15635	–	188	–	<i>P. putida</i> S16, <i>P. monteilii</i> SB3078
HNH-HHH nuclease	1385–1392	PF15637	–	153–158	–	<i>P. putida</i> KT2440, <i>P. syringae</i> pv. <i>syringae</i> SM
HNH-LHH nuclease	484–688	PF14411	SUKH-6	156	PF14568	<i>P. putida</i> DOT-T1E, <i>P. syringae</i> BRIP34881
HNH-SHH nuclease	1408–1530	PF15652	SUKH-1	161–197	PF09346	<i>P. putida</i> KT2440, <i>P. plecoglossicida</i> NB2011
HNH-WHH nuclease	1386–1472	PF14414	SUKH-1	136–157	PF09346	<i>P. brassicacearum</i> subsp. <i>brassicacearum</i> NFM421, <i>P. fluorescens</i> F113, <i>P. fluorescens</i> Q2–87, <i>P. poae</i> RE*1-1-14, <i>P. putida</i> W619
HYD1 ADP-ribosyltransferase	1534–1537	PF15633	–	97–212	–	<i>P. poae</i> RE*1-1-14, <i>Pseudomonas</i> sp. TKP
NS endonuclease	1407–1599	PF13930	DUF1963	220–222	PF09234	<i>P. chlororaphis</i> subsp. <i>aureofaciens</i> 30–84, <i>Pseudomonas</i> sp. GM41
			NTF2 fold	97–150	PF15655	<i>P. syringae</i> BRIP34881, <i>P. syringae</i> pv. <i>glycinea</i> B076, <i>P. syringae</i> pv. <i>syringae</i> B64, <i>Pseudomonas</i> sp. GM55, <i>Pseudomonas</i> sp. VLB120
ParB-like nuclease	1511–1566	PF02195	DUF260	126–136	PF03195	<i>P. entomophila</i> L48, <i>P. fluorescens</i> A506, <i>Pseudomonas</i> sp. CFT9
Pput2613-deaminase	866–1560	PF14427	Imm7	127–131	PF15588	<i>P. entomophila</i> L48, <i>P. putida</i> ND6
Toxin 61	1631	PF15538	–	90	–	<i>P. fluorescens</i> NCIMB 11764
URI nuclease	898–1562	PF15653	DUF2384	93–95	PF09722	<i>P. fluorescens</i> SBW25, <i>P. mandelii</i> 36MFCvi1.1
			Imm25	135–144	–	<i>P. fluorescens</i> BBc6R8, <i>Pseudomonas</i> sp. Ag1, <i>Pseudomonas</i> sp. VLB120
YwqJ-like deaminase	1561	PF14431	–	120	–	<i>Pseudomonas</i> sp. GM80

GenBank Accession Numbers are provided in Table S2.

*Product of gene located immediately downstream of toxin gene.

†Based on domain present in CT module; –, not assigned.

‡Number of amino acids or size range of different proteins.

§Pfam (PF) or SMART (SM); –, not defined.

Structure and domain organization of lectin-like bacteriocins

The first identified member of this novel family of antibacterial proteins was called LlpA, abbreviation for lectin-like putidacin A and referring to its remarkable sequence homology with monocot mannose-binding lectins (MMBLs) from plants (Parret *et al.*, 2003). In the latter organisms, these carbohydrate-binding proteins have

been associated with a number of antimicrobial activities, such as antifungal, antiviral, or nematocidal action, though no antibacterial plant MMBL has been identified at this point (Ghequire *et al.*, 2012a). The amino acid sequence of LlpA suggests the presence of a tandem of such MMBL domains, followed by a short, highly variable carboxy-terminal extension that does not occur in plant MMBLs (Parret *et al.*, 2003; Van Damme *et al.*, 2008). Monocot mannose-binding lectin domains typically adopt

a β -prism fold, stabilized by a conserved tryptophan triad, and harbor three potential carbohydrate-binding pockets, each set up by a conserved QxDxNxVxY sequence motif (with x, any amino acid). However, motif degeneracy suggests that several of these sites may be inactive in LlpAs (Ghequire *et al.*, 2012a; McCaughey *et al.*, 2014). The size of the MMBL domain is c. 12 kDa, resulting in a rather narrow molecular weight range of 28–32 kDa for LlpAs, contrary to S-type pyocins where considerable size variation is observed (Table 1).

The crystal structure of the prototype LlpA from *P. putida* BW11M1 revealed a rigid, tightly interconnected, tandem MMBL structure sharing unequivocal similarities with plant MMBLs (Fig. 10; Parret *et al.*, 2004; Ghequire *et al.*, 2013a). More recently, the structure of pyocin L1, a distant LlpA homolog from *P. aeruginosa* C1433 sharing c. 27% amino acid identity, was elucidated, displaying a similar lectin architecture but apparently more closely related to dimeric plant lectins due to a different relative orientation of the MMBL modules (McCaughey *et al.*, 2014). Loyal to their protein family, these LlpAs were found to act as genuine lectins though binding to mannose and oligomannosides is rather weak (Ghequire *et al.*, 2013a; McCaughey *et al.*, 2014), hinting to another preferred carbohydrate ligand. Recently it was found that these tandem MMBLs bind D-rhamnose with higher affinity (McCaughey *et al.*, 2014). Consistent with this, binding to CPA, the *P. aeruginosa* common polysaccharide antigen that is predominantly composed of D-rhamnosyl residues (Lam *et al.*, 2011), was demonstrated. Furthermore, lipopolysaccharide mutants in CPA

biosynthesis genes were shown to be almost insensitive to pyocin L1. Further support for D-rhamnose-containing lipopolysaccharide acting as a receptor for these MMBL lectins stems from the capacity of *P. putida* LlpA to bind lipopolysaccharide of sensitive *P. syringae* cells (McCaughey *et al.*, 2014). The ability to bind carbohydrates could only be attributed to the second, carboxy-terminal MMBL domain. The construction of *P. putida*–*P. protegens* chimeric LlpA forms revealed that the amino-terminal MMBL module hosts the target specificity function (Ghequire *et al.*, 2013a).

Occurrence of MMBL bacteriocins

Originally identified in a *P. putida* rhizosphere isolate, whole-genome sequencing projects have revealed a number of putative LlpAs in other *Pseudomonas* strains. The occurrence of *llpA* sequences seems biased toward plant- and soil-associated isolates, and it is tempting to speculate about plant-to-bacterium transfer of the ancestral prokaryotic genes (Ghequire *et al.*, 2012a; Loper *et al.*, 2012). Next to LlpA from *P. putida* BW11M1, narrow-spectrum antibacterial activity was also assigned to two proteins in biocontrol strain *P. protegens* Pf-5, called LlpA1 and LlpA2 (Fig. 1), with near identical amino acid sequences and indistinguishable target spectrum (Parret *et al.*, 2005). More recently, a *P. syringae* LlpA (Ghequire *et al.*, 2012b) and a *P. aeruginosa*-specific LlpA (Fig. 1; McCaughey *et al.*, 2014) were demonstrated to act as functional bacteriocins as well. One peculiar characteristic of these LlpAs is their overlapping activity spectrum: a strain

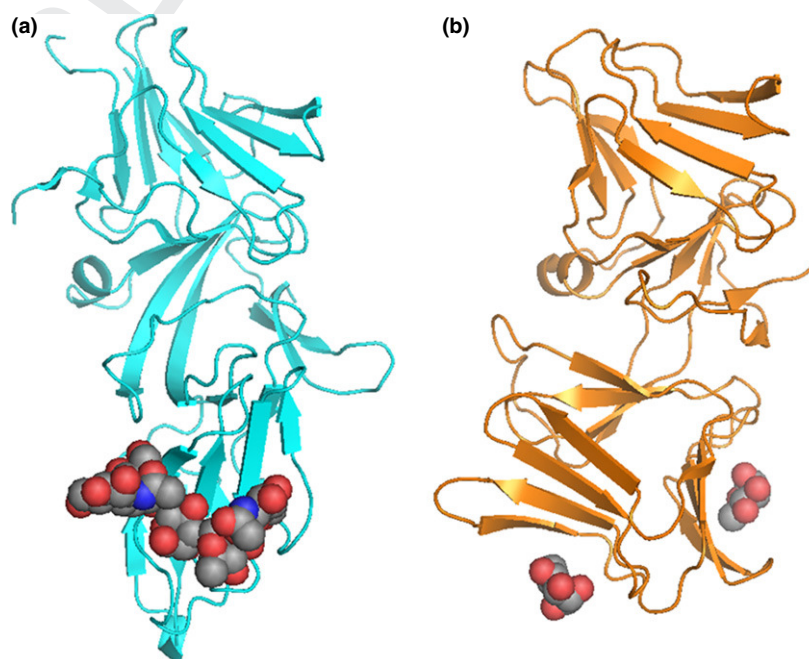


Fig. 10. Ribbon structures of lectin-like bacteriocins *Pseudomonas putida* LlpA (a, PDB 4GC2) and *Pseudomonas aeruginosa* pyocin L1 (b, PDB 4LED). Oligomannoside (GlcNAc β (1–2)Man α (1–3)[GlcNAc β (1–2)Man α (1–6)]Man) in complex with LlpA and D-rhamnose residues in complex with pyocin L1 are shown as spheres bound to the respective carboxy-terminal MMBL domains.

may be sensitive to more than one LlpA, even if sequence homology between the amino-terminal domains of the LlpAs involved is barely present. Tandem-MMBL bacteriocins were also retrieved in two *Xanthomonas* pathovars (Ghequire *et al.*, 2012b) and in *Burkholderia cenocepacia* (Ghequire *et al.*, 2013b), equally displaying genus-specific activity.

The sequence divergence among currently known LlpA proteins is visualized in Fig. S10. The most distant *Pseudomonas* sequences share only 27% amino acid sequence identity, mostly focused around the MMBL motifs and the MMBL-stabilizing tryptophan triads. No obvious correlation is apparent between the LlpA sequence-based phylogeny and taxonomic relatedness of the respective producers, suggesting LlpAs to act as highly diversified niche-specific molecular weapons. Considering the limited number of identified *llpA* genes, it appears that LlpA production is relatively rare among pseudomonads, compared to the abundance of pyocin-like bacteriocins. The *llpA* clusters from recently sequenced *P. aeruginosa* genomes extend the occurrence of these antimicrobials to a human pathogenic pseudomonad. At this point, it remains unclear how MMBL bacteriocins are released by their producer strains as *Pseudomonas* LlpAs are typically not preceded by a cleavable signal sequence. Exception to this are the proteins encoded by one of the *P. aeruginosa* clusters, apparently following a different Sec-dependent route (Fig. S10).

Phylogenetic analysis of individual MMBL modules unveiled a clustering of the amino-terminal MMBL domains, separate from clustered branches with carboxy-terminal domains (Ghequire *et al.*, 2012a). The latter is consistent with the domain-function segregation demonstrated for *P. putida*–*P. protegens* LlpA chimeras (Ghequire *et al.*, 2013a). Higher sequence conservation of the carboxy-terminal MMBL may be imposed by binding to a common lipopolysaccharide moiety. Conversely, the amino-terminal domains evolved rather independently to define diversified target spectra.

Genome mining of MMBL domains among pseudomonads also revealed a second type of putative lectin-like bacteriocin (Ghequire *et al.*, 2012a), which we call LlpB (Fig. 1). Consisting of only a single MMBL domain, these LlpBs equally carry a poorly conserved carboxy-terminal extension. Initial assays with recombinantly produced LlpBs indicate that these proteins are functional bacteriocins as well (Ghequire, 2013).

Lectin-like bacteriocins as a cargo in *Pseudomonas* prophages

Similar to pyocin M2 and some of its homologs, a number of lectin-like bacteriocins are also encoded by cargo

genes of putative prophages. In *P. syringae* pv. *syringae* 642, this is the case for both a pyocin M2 and a LlpA homolog, albeit in different prophages and unlinked genomic locations. A similar prophage carrying a *llpA*-like gene is present in the *Pseudomonas* sp. GM80 genome, whereas its counterpart in *P. fluorescens* A506 has taken up an *llpB* gene (Fig. 1). In *Pseudomonas* sp. GM80, a second distinct *llpA* gene is equally located on a prophage that has inserted in the *mutS*–*cinA* intergenic region and the latter configuration is also found in *P. chlororaphis* subsp. *aureofaciens* 30–84 for yet another *llpA* gene. Like strain GM80, *P. protegens* Pf-5 also displays a dual LlpA bacteriocinogeny (Parret *et al.*, 2005). LlpA1 of this strain is encoded by a cargo gene on prophage-1, equally integrated between the *mutS* and *cinA* genes, whereas its *llpA2* gene is located in the defective prophage-4 region (Mavrodi *et al.*, 2009). The *llpA2* gene is also present in *P. protegens* CHA0, a strain closely related to Pf-5 (Jousset *et al.*, 2014), but only a short prophage-4 remnant remains, apparently due to a c. 10-kb deletion in the *llpA2* upstream region. The *llpA1* gene is lacking in this strain. Actually, its *mutS*–*cinA* hotspot has been targeted by the same type of *llpA*-carrying phage as present in *Pseudomonas* sp. GM80 and *P. chlororaphis* subsp. *aureofaciens* 30–84, however, without bringing an *llpA* cargo. The high sequence similarity between LlpA1 and LlpA2 of *P. protegens* Pf-5, exhibiting indistinguishable activity spectra (Parret *et al.*, 2005), suggests that *llpA1* may have been acquired more recently and is maintained to enable further divergence of the both bacteriocin genes toward a broader antagonistic spectrum.

Regulation of LlpA synthesis

At this point, only limited information is available on the regulation of *llpA* genes. Constitutive expression has been observed for the LlpAs of *P. putida* BW11M1 and *P. protegens* Pf-5 (Parret *et al.*, 2003, 2005), and – similar to S-type pyocins – LlpA_{BW11M1} production is enhanced upon exposure to UV light (Parret *et al.*, 2003). For the *P. putida* bacteriocin, reduced production was detected upon *recA* and *spoT* inactivation, similar to S-type pyocins (see Regulation of pyocin expression in *P. aeruginosa*). Conversely, a *recJ* mutant exhibited an overexpression phenotype (de los Santos *et al.*, 2005).

Thiazole-/oxazole-modified microcins

Microcin B-like bacteriocins of *P. syringae*

In contrast to their capacity to produce very diverse secondary metabolites (Gross & Loper, 2009), pseudomonads are not endowed with elaborate tools for post-translational

modification of ribosomally synthesized peptides (Arnison *et al.*, 2013). Recently, the genetic capacity to produce B-type microcins was however demonstrated for strains of the *P. syringae* pathovars *glycinea* and *aesculi* by genome exploration with genes of the *E. coli* microcin B17 (*Ec*-Mcb) operon *mcbABCDEFGHI* as queries (Metelev *et al.*, 2013). The DNA gyrase inhibitor microcin B17 is derived from the ribosomally synthesized McbA precursor by extensive post-translational processing (reviewed by, Duquesne *et al.*, 2007; Severinov *et al.*, 2007). The McbA leader peptide drives substrate binding by the MbcBCD complex that converts selected serine and cysteine residues to azoles or thiazoles by consecutive MbcCD-mediated ATP-dependent cyclodehydration (Dunbar *et al.*, 2012) and McbB-catalyzed dehydrogenation. The unmodified leader peptide is proteolytically removed by conserved enzymes (TldD, TldE) prior to export by the dedicated ABC transporter MbcEF. Both this transporter and MbcG contribute to self-immunity of the microcin B-producing *E. coli* cells.

In two *P. syringae* strains, an equivalent *mcbABCDEFGHI* operon was identified, but production of a microcin-like compound was not detected, albeit active *mcb* transcription was demonstrated (Metelev *et al.*, 2013). However, two biologically active gyrase-targeting microcins, *Ps*-Mcb1 (minor product) and *Ps*-Mcb2 (major product with the five McbA carboxy-terminal amino acids lacking), were produced by *E. coli* carrying the *P. syringae* *mcb* gene cluster. Two *mcb*-lacking *P. syringae* strains and *P. aeruginosa* PAO1 were found susceptible to both *Ps*-Mcb. While these pseudomonads are not susceptible to *Ec*-Mcb, *P. syringae* microcin exhibits anti-*E. coli* activity. By using chimeric *mcbA* genes, Metelev *et al.* (2013) identified a centrally located unmodified tri-glycine moiety as a key determinant for activity against the pseudomonads, apparently required for uptake by a specific, as yet unidentified transporter.

Microcinogenic potential of pseudomonads

Scrutiny of currently available genome sequences of pseudomonads indicates that several strains of different species are potential producers of microcin B-like bacteriocins, as inferred from the presence of an equivalent *mcbABCDEFGHI* cluster, however, consistently lacking *mcbG* (Fig. 1). The *mcb* clusters, which are often incompletely annotated, are not located in a particular genomic region of these strains. The azole-triazole pattern in mature *Ec*-Mcb and *Ps*-Mcb peptides is essentially the same, but more diversity among putative B-type microcins from other *Pseudomonas* strains is potentially present in the deduced McbA amino acid sequences (Fig. 11). On both sides of the conserved central glycine triplet, the presence of potential modification sites allows for patterns different from those of *E. coli* and *P. syringae* microcins. The sequence divergence is also apparent for the respective leader sequences. This trend is reflected in the phylogenetic relationship among the cognate synthetase and transporter proteins, indicating that, unexpectedly, the *P. syringae* microcin assembly system is actually more similar to the *E. coli* machinery (including the presence of the MbcG protein) in comparison to the putative Mbc proteins in other pseudomonads. No equivalent *mcb* gene cluster was found in one of the many available *P. aeruginosa* genome sequences. It appears that microcin production is relatively rare among pseudomonads compared with the abundance of pyocin-like bacteriocins.

General discussion

Current knowledge on pseudomonad bacteriocins is largely based on early study carried out with *P. aeruginosa* to characterize the antagonistic behavior observed among strains of this pathogenic species. When this aspect of pseudomonad sociomicrobiology was reviewed by

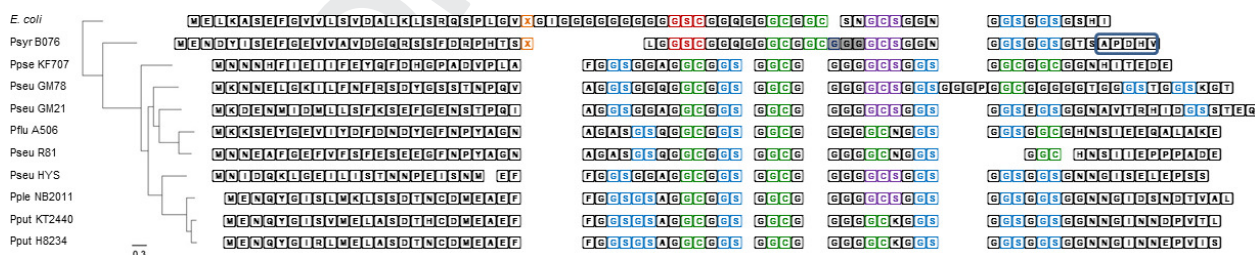


Fig. 11. Microcin B-like bacteriocins in pseudomonads. The ML phylogenetic tree (left) is inferred from multiple alignment of concatenated *Pseudomonas* and *Escherichia coli* MbcBCDEF amino acid sequences. Cleavage sites (orange X) of the leader sequences in the *E. coli* MccB17 precursor and the *Pseudomonas syringae* *Ps*-Mcb peptides, shown in the multiple alignment of McbA sequences (right), are indicated. Sites with amino acid pairs or triplets subject to (potential) posttranslational modifications are highlighted in color: G-C (thiazole; green), G-S (oxazole; blue), G-C-S (fused thiazole-oxazole; purple), G-S-C (fused oxazole-thiazole; red). The pentapeptide lacking in *Ps*-Mcb2 is boxed. The central G-G-G motif of *Ps*-Mcb is shown on a gray background.

Michel-Briand and Baysse (Michel-Briand & Baysse, 2002), two main different types of DNA-degrading S-type pyocins and two main sorts of phage tail-like particles (R and F pyocins) had been identified. Subsequently, cytotoxic activities that were initially discovered in colicins from *E. coli*, targeting RNA or disrupting cell envelope integrity, were also assigned to S-type pyocins. However, insights in the biology of these pseudomonad bacteriocins is lagging behind the colicin field, in particular with respect to structural characterization which is instrumental to elucidate how these proteins reach their target. Some recent additions to the armamentarium of pseudomonads are B-type microcins and lectin-like bacteriocins, the latter not (yet) known to be produced by enterobacteria.

Inspection of currently available pseudomonad genome sequences provides a glimpse inside the bacteriocin profiles of these bacteria and highlights the strain-specific nature of bacteriocinogeny. Even within the *P. aeruginosa* species, considered to be a phylogenetically well-defined entity, few strains display the same bacteriocinogenic profile. This is illustrated in Fig. 12 in which the known bacteriocins of the model strain *P. aeruginosa* PAO1 are compared with 52 different bacteriocin gene combinations predicted for individual strains. In *P. aeruginosa*, the systems acting on nucleic acids (DNA or RNA) predominate together with the widespread tailocins. The latter class of bacteriocins, evolutionary related to phages, is relatively rare among other *Pseudomonas* species and is confined to two subtypes of R-like pyocins. These predicted tailocin systems differ from their *P. aeruginosa* counterparts not only in the host specificity determinants and lysis cassettes used but also in the regulatory modules. Furthermore, these mobile units are integrated at a different but specific genomic location and seem apt to expansion with cargo genes. Certain bacteriocin genes are also loaded onto prophages, as noted for some members of the pyocin M and LlpA families. Such hitchhiking is reminiscent of prophage-carried eukaryote-targeting toxins such as Stx and Dtx (diphtheria toxin) that can assist in defense of their bacterial-host population against bacteria-predating amoebae (Arnold & Koudelka, 2014). The assembly and fine structure of pseudomonad tailocins is an unexplored field and application of a structural biology approach, as currently used to elucidate the intricacies of the T6S toxin delivery system – also sharing features with certain phages – is likely to provide new insights in the mode of action of this killing machinery.

Categorization of S-type pyocins based on their carboxy-terminal cytotoxic domains further highlights their dynamic modular architecture. It was already described in *P. aeruginosa* that the same receptor-recognition domain can be fused to a different HNH-type DNase domain

present in pyocins S1 and S2. Genomic analysis shows that certain receptor-recognition domains are also polyvalently employable with toxin modules of a different nature (Fig. 1). Such domain sharing occurs between a HNH DNase and a rRNase in both *P. aeruginosa* and *P. chlororaphis*, and between a non-HNH DNase and a tRNase among *P. aeruginosa* strains. Combinations of a HNH DNase, a non-HNH DNase or a rRNase with a particular receptor-recognition domain are found in different *P. aeruginosa* strains. Proof-of-principle that this modularity can be exploited to engineer bacteriocins with novel target specificities or equipped with a different type of warhead has already been delivered. The functionality of certain pyocin–colicin chimeras shows that this mimicry of Nature's recombinational strategy is not constrained by genus limits. A conceptually similar strategy can even be applied to tailocins by transposing insights from phage biology to these complex bacteriocins, enabling the construction of inter-generic tailocin hybrids with new functionalities by exchange of tail fiber genes.

Some novel features not associated with *P. aeruginosa* bacteriocins emerge from the genomic perspective on bacteriocinogeny broadened to other members of the *Pseudomonas* genus. This highlights the huge genetic diversity contributed by other pseudomonad species, being most prominent for strains originating from plant and soil environments (Fig. 13). Microcin-encoding gene clusters were not yet identified in *P. aeruginosa* but are present in several other species. Although nucleic acids are equally the preferred target in species other than *P. aeruginosa*, the widespread occurrence and broad diversity of predicted rRNase bacteriocin genes, encoding a novel type of cytotoxic activity only recently described for *P. aeruginosa* and present in only few of its strains, is striking. Certain strains dispose of an expanded offensive arsenal composed of multiple bacteriocins, in particular enzymes of the HNH DNase and rRNase types. On the other hand, strains tend to enlarge their defensive capacity against intruding toxins from competitors by expanded immunity loci or acquisition of orphan immunity genes. Of particular interest are three different types of hybrid genes that encode putative bacteriocins with two different cytotoxic modules integrated in a single polypeptide. Conceivably, such dual mode of action may enhance the efficacy of a bacteriocin-mediated strike, as tolerance of the attacked to two different cytotoxic agents is less likely.

Whereas little is known how various types of pyocins are secreted, studies with *P. aeruginosa* have contributed substantially to recognition of the important role played by T6S-translocated effector molecules in antagonistic interactions with rival bacteria. Though not yet well understood in pseudomonads, conceptually similar strategies, making use of T5S-translocated CDI-mediating substances and of

LOW RESOLUTION COLOR FIG

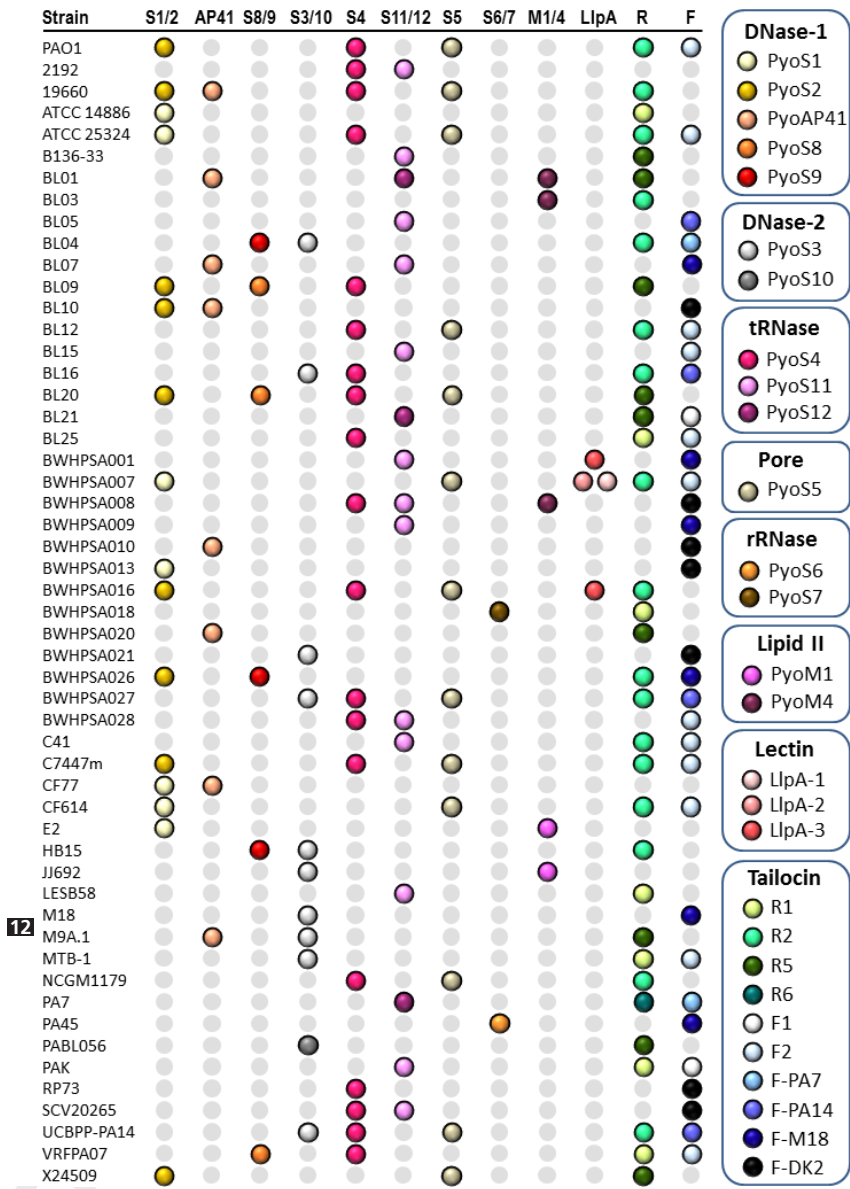


Fig. 12. Bacteriocin profiles of *Pseudomonas aeruginosa* strains. The bacteriocin complement of strain PAO1 is compared to the predicted bacteriocin profiles of representative strains carrying genes or gene clusters for at least two different types of bacteriocins. The presence of a particular type of bacteriocin gene or gene cluster, classified by assigned/predicted toxic domain or mode of action, is indicated by colored dots.

Rhs protein release, appear to be at hand for pseudomonads. The modular nature of pyocins is reflected in the general architecture of CdiA and Rhs proteins. Apparently, these three classes of toxins share certain types of cytotoxic modules, along with cognate self-protective immunity proteins. Whereas most predicted pyocin gene products can be assigned to a known group of pyocins or colicins, the equally carboxy-terminally located cytotoxic modules seem to have diversified to a much larger extent in CdiA proteins and Rhs proteins, warranting their classification as polymorphic toxins. With only few of such toxin modules being functionally characterized and many escaping sequence homology-based familial assignment, it is clear that a considerable part of the ribosomally encoded armory

remains to be uncovered. Nevertheless, it seems that the use of the nucleic acid-targeting strategy, dominating S-type pyocin action, is also deployed frequently with these polymorphic toxins. The functionality of many of the bacteriocin genes described in this review, including divergent members of known groups as well as representatives of novel groups, requires experimental validation. Often, expression of bacteriocin genes in a natural host cannot be provoked in laboratory conditions. Hence, identification of the environmental factors triggering production of bacteriocins is another imperative toward assessment of their ecological role in bacterial rivalry and of their impact on populations of related bacteria favoring similar niches.

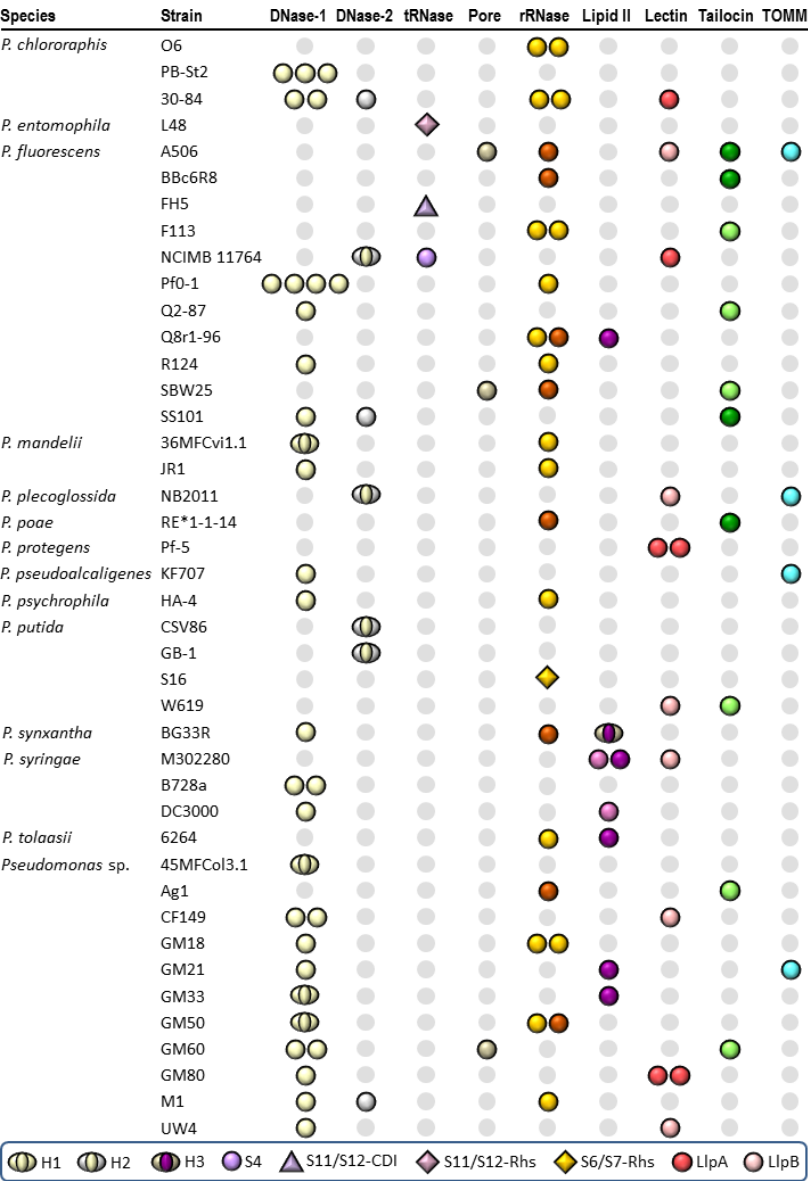


Fig. 13. Bacteriocin profiles of pseudomonads other than *Pseudomonas aeruginosa*. The predicted bacteriocin complement of representative strains carrying genes or gene clusters for at least two different bacteriocins are shown. The presence of a particular type of bacteriocin gene or gene cluster is indicated by a colored dot. Multiple dots of the same color reflect the potential to produce two to four bacteriocins of a particular toxin type. The pyocin classification is based on similarity of the putative toxic domain or mode of action to *P. aeruginosa* bacteriocins: DNase-1 (pyocins S1, S2, AP41, S8, and S9); DNase-2 (pyocins S3 and S10); tRNase (pyocin S4); pore-forming toxin (pyocin S5); rRNase (pyocin S6 and S7). In addition, strains encoding a putative Rhs or CDI protein with a toxic domain similar to pyocins are included (triangle or diamond symbol). Proteins with a rRNase domain phylogenetically clustering with the toxic domain of *P. aeruginosa* pyocin S6/ S7 (light orange dots) are distinguished from those constituting a separate cluster (darker orange dots). Putative lipid II-targeting toxins distinct from *Pseudomonas syringae* syringacin M (PyoM2; light purple dots) are marked with a different color (darker purple dots). A different shade of green denotes the two main types of R-pyocin-like gene clusters (see Fig. 6). Gene clusters encoding TOMM-type microcins are similar to the *P. syringae* pv. *glycinea* B076 *mcb* operon. The eye symbols denote hybrid forms with two toxic domains (H1–H3; discussed in the text).

LOW RESOLUTION COLOR FIG

The stimuli that activate bacteriocin-mediated warfare may differ among particular niches, such as plant rhizospheres colonized by saprophytic bacteria or eukaryotic host tissues invaded by pathogenic, symbiotic, or endophytic bacteria.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phylogenetic analysis of HNH DNase pyocin immunity proteins.

Fig. S2. Phylogenetic analysis of the cytotoxic domains of non-HNH DNase pyocins.

Fig. S3. Phylogenetic analysis of non-HNH DNase pyocin immunity proteins.

Fig. S4. Phylogenetic analysis of cytotoxic domains of bacteriocins with a colicin D-like tRNase domain.

Fig. S5. Phylogenetic analysis of colicin D-like immunity proteins.

Fig. S6. Phylogenetic analysis of immunity proteins associated with pyocin S6-like rRNase bacteriocins.

Fig. S7. Sequence alignment of amino-terminal regions of lipid II-degrading M pyocins.

Fig. S8. Phylogenetic analysis of tail fiber proteins of F pyocins.

Fig. S9. Sequence alignment of CdiA-CT tRNase domains.

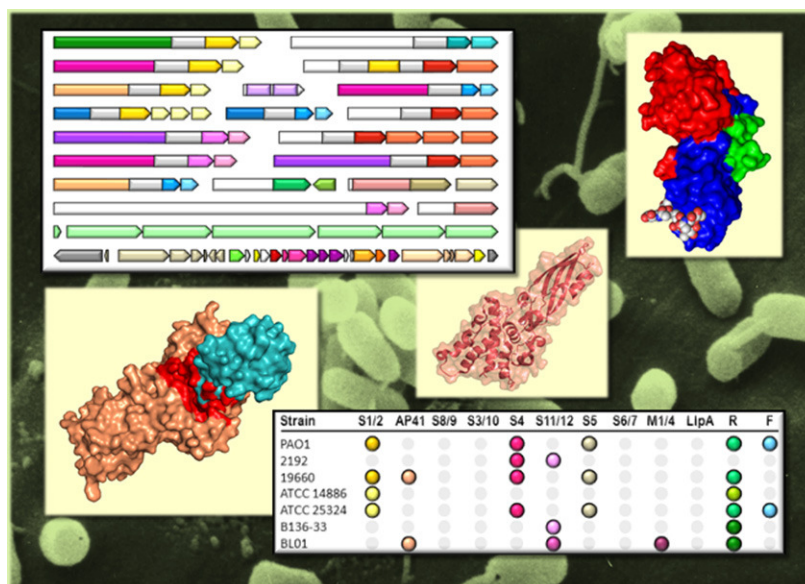
Fig. S10. Phylogenetic analysis of tandem-MMBL bacteriocins.

Table S1. Bacteriocin/pyocin genes present in genome sequences of pseudomonads.

Table S2. Genes encoding Rhs or CDI proteins present in genome sequences of pseudomonads.

Graphical Abstract

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The past decade, knowledge on secreted and contact-dependent *Pseudomonas* peptides and proteins exhibiting antibacterial activity has expanded enormously, warranting a timely update of this aspect of sociomicrobiology for *Pseudomonas aeruginosa* and other *Pseudomonas* species.

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